

CORRECTED VERSION

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
20 April 2000 (20.04.2000)

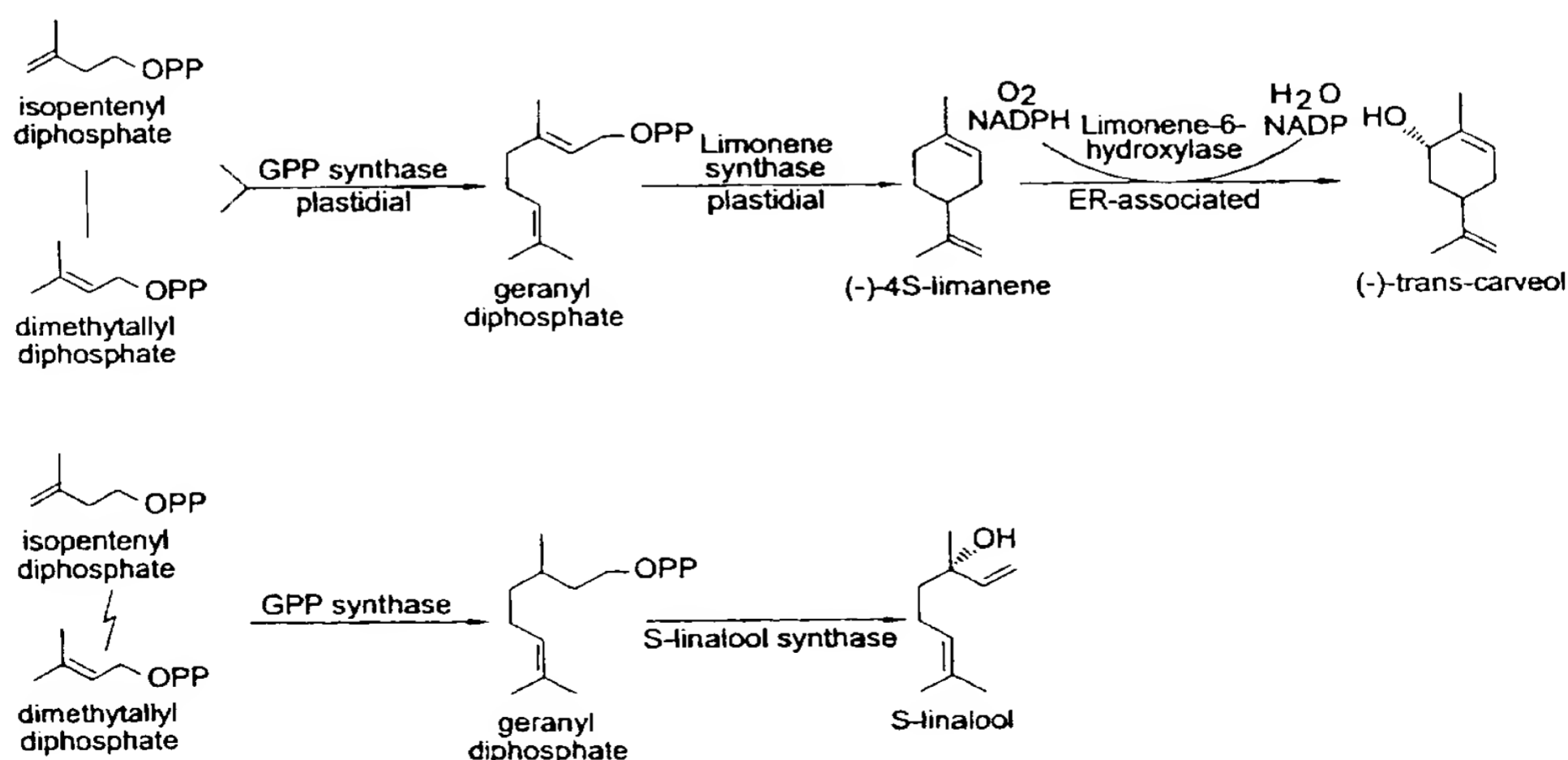
PCT

(10) International Publication Number  
WO 00/22150 A3

- (51) International Patent Classification: C12N 15/82, 9/88, 9/10, 9/00, 15/11, A01H 5/00
- (21) International Application Number: PCT/US99/23180
- (22) International Filing Date: 5 October 1999 (05.10.1999)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
09/172,339 14 October 1998 (14.10.1998) US
- (63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:  
US 09/172,339 (CON)  
Filed on 14 October 1998 (14.10.1998)
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- (81) Designated States (national): AE, AL, AM, AT, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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(54) Title: LIMONENE AND OTHER METABOLITES OF GERANYL PYROPHOSPHATE FOR INSECT CONTROL



(57) Abstract: Methods for manipulating metabolic pathway in plants, particularly those pathways involved in the biosynthesis of monoterpenes are provided. Methods are directed at transforming plants with one or more nucleotide sequences encoding the enzyme GPP synthase, and the monoterpene synthases limonene-, carveol and S-linalool synthase. Methods for creating or enhancing resistance to insects in plants by transforming plants with GPP- and/or monoterpene synthases, to generate plants producing monoterpenes in amount effective for resistance to insects are also provided.



WO 00/22150 A3



**Published:**

— *With international search report.*

**(15) Information about Correction:**

see PCT Gazette No. 10/2001 of 8 March 2001, Section II

**(88) Date of publication of the international search report:**

5 October 2000

**(48) Date of publication of this corrected version:**

8 March 2001

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

## LIMONENE AND OTHER METABOLITES OF GERANYL PYROPHOSPHATE FOR INSECT CONTROL

## FIELD OF THE INVENTION

This invention relates to methods for genetic manipulation of metabolic pathways in plants, particularly to transforming plants with genes involved in monoterpene biosynthesis and resistance to insects.

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## BACKGROUND OF THE INVENTION

Numerous insects are serious pests of common agricultural crops. One method of controlling insects has been to apply insecticidal organic, semiorganic or organometallic chemicals to crops. This method has numerous, art-recognized environmental and public health problems. A more recent method of control of insect pests has been the use of biological control organisms which are typically natural predators of the troublesome insects. These include other insects such as trachonid wasps, fungi such as *Beauveria Bassiana*, and bacteria such as *Bacillus thuringiensis* cv., commonly referred to as "Bt". However, it is difficult to apply biological control organisms to large areas, and even more difficult to cause those living organisms to remain in the treated area for an extended period. Still more recently, techniques in recombinant DNA have provided the opportunity to insert into plant cells cloned genes which express insecticidal toxins derived from biological control organisms such as Bt. This technology has given rise to concerns about eventual insect resistance to well-known, naturally occurring insect toxins, particularly in the face of heavy selection pressure, which may occur in some areas. Thus, a continuing need exists to identify naturally occurring insecticidal toxins which can be formed by plant cells directly by expression of structural genes not normally present in the plant.

25        Southern Corn Rootworm (*Diabrotica undecimpunctata howardi* Barber) is a particularly difficult pest to control or eradicate. It attacks the plant below the soil line, where insecticides are difficult or impossible to apply effectively. In addition, it is resistant to a number of otherwise effective chemical and biological control agents, including Bt toxins and some lectins.

The monoterpene, limonene, 1-methyl-4 (1-methylethenyl) cyclohexene; p-mentha-1,8-diene (Entry No. 5371, Merck Index 11th Ed ), occurs naturally in various ethereal oils, particularly oils of lemon, orange, caraway, dill and bergamot. It is a valuable industrial chemical. Some limonene is prepared by  
5 extraction from plants of the mint family, a large quantity is obtained from citrus oils, which are typically 80-90% limonene, and some is obtained from pine oil. It is also synthesized chemically and finds use as a solvent and cleaning agent (in the manufacture of synthetic pine oil), as an expectorant, as a wetting and dispersing agent, as a monomer in the manufacture of various polymeric resins, as a flavorant  
10 and a precursor in the synthesis of the flavorant carvone, and as a polymerization inhibitor in storage of the tetrafluoroethylene monomer used in the manufacture of polytetrafluoroethylene (PTFE).

Geranyl diphosphate (GPP) synthase catalyzes the first committed step of monoterpene biosynthesis by the condensation of dimethylallyl diphosphate  
15 (DMAPP) and isopentenyl diphosphate (IPP) to form GPP, the immediate acyclic precursor of monoterpenes. GPP is converted to (-)-4S-limonene by the catalytic action of (-)-4S-limonene synthase (cyclase). (-)-4S-limonene is converted to (-)-trans-carveol by the action of (-)-trans-carveol synthase, also referred to as limonene-6-hydroxylase. (-)-trans-carveol is converted to carvone by the action of  
20 (-)-trans-carveol dehydrogenase, also referred to as carvone synthase. GPP is also converted to the monoterpene S-linalool by the action of S-linalool synthase. Thus, GPP is the precursor of (-)-4S-limonene and its downstream metabolites (-)-trans-carveol and carvone; as well as the precursor of S-linalool. See Figure 1; Wise *et al.* (1997) In "*Comprehensive Natural Products Chemistry: Isoprenoids*,  
25 *Vol. 2*" (Cane, D.E., ed.), Elsevier Science, Oxford (in press); Gershenzon *et al.* (1989) *Plant Physiol.* 89:1351-1357; Pichersky *et al.* (1994) *Plant Physiol.* 106:1533-1540. Unlike the mechanistically-related prenyltransferases farnesyl diphosphate (FPP) synthase and geranylgeranyl diphosphate (GGPP) synthase, which produce GPP as intermediates and which are nearly ubiquitous (Ogura *et al.*  
30 (1997) In "*Dynamic Aspects of Natural Products Chemistry*" (Ogura, K. and Sankawa, U., eds.), Kodansha/Harwood Academic Publishers, Tokyo, pp. 1-23), GPP synthase is largely restricted to plant species that produce abundant quantities of monoterpenes.

Because both farnesyl diphosphate synthase and geranylgeranyl diphosphate synthase produce only negligible levels of GPP as a free intermediate on route to FPP and GGPP (Ogura *et al.* (1997) In "*Dynamic Aspects of Natural Products Chemistry*" (Ogura, K. and Sankawa, U., eds.), Kodansha/Harwood Academic Publishers, Tokyo, pp. 1-23), it is geranyl diphosphate synthase that provides the crucial link between primary metabolism and monoterpene biosynthesis and that serves as the essential driver of monoterpene biosynthesis (Wise *et al.* (1997) In "*Comprehensive Natural Products Chemistry: Isoprenoids, Vol. 2*" (Cane, D.E., ed.), Elsevier Science, Oxford (in press)).

GPP synthase has been isolated from several plant sources, including grape, geranium, sage (Croteau *et al.* (1989) *Arch. Biochem. Biophys.* 271:524-535; Heide *et al.* (1989) *Arch. Biochem. Biophys.* 273:331-338; Suga *et al.* (1991) *Phytochemistry* 30:1757-1761; Clastre *et al.* (1993) *Plant Physiol.* 102:205-211); however, the enzyme has not been characterized in any detail, and only the enzyme from grape has been purified to homogeneity.

A cDNA encoding 4S-limonene synthase from oil glands of spearmint has been described in Colby *et al.* (1993) *J. Biol. Chem.* 268(31): 23016-23024 and is available in the Genbank<sup>TM</sup>/EMBL database and identified by the accession number L13459.

cDNAs encoding S-linalool synthase from *Clarkia Breweri* have been described in WO 97/15584, along with methods directed at using the cDNA for enhancing the flavor and smell of plants; and in Dudareva *et al.* (1996) *Plant Cell* 8 (7):1137-1148, also available in the Genbank<sup>TM</sup>/EMBL database and identified by the accession number 1491939.

To exploit recombinant methods to increase monoterpene yield in monoterpene-producing species, or to genetically engineer the monoterpene biosynthetic pathway into non-producing species, it would be highly beneficial to manipulate a GPP synthase gene. Accordingly, the invention relates to expressing GPP synthase in combination with selected monoterpene synthases such as (-)-limonene synthase, S-linalool synthase, and subsequent and related pathway enzymes for production of the corresponding monoterpene product(s).

## SUMMARY OF THE INVENTION

The invention provides methods for manipulating metabolic pathways in plants, particularly those pathways that are involved in the biosynthesis of  
5 monoterpenes. Methods are directed at transforming plants, plant tissues and cells with one or more nucleotide sequences encoding the enzyme GPP synthase, and the monoterpene synthases limonene-, carveol and S-linalool synthase.

Methods are also provided for creating or enhancing resistance to insects in plants by transforming plants with GPP- and/or monoterpene synthases, to generate  
10 plants producing monoterpenes in amounts effective for resistance to insects.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the biosynthetic pathways involved in production of the monoterpenes limonene, carveol, and S-linalool.  
15

Figure 2 schematically illustrates the plasmid construct comprising the ubiquitin promoter and a monoterpene synthesis pathway enzyme.

Figure 3 schematically illustrates the plasmid construct PHP14099 for  
20 expression of GPP- and S-linalool synthases, and production of S-linalool in plants.

Figure 4 schematically illustrates the plasmid construct PHP14100 for  
25 expression of GPP-, limonene- and carveol synthases; and production of limonene and carveol.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods for manipulating metabolic pathways in plant cells; particularly those pathways related to synthesis of  
30 monoterpenes including but not limited to the monoterpenes (-)-4S-limonene (herein limonene), (-)-trans-carveol (herein carveol) and S-linalool in plants, plant cells and specific plant tissues. These monoterpenes have insecticidal and/or repellent activity against insect pests.

Accordingly, the invention provides methods useful for increasing monoterpene yield in monoterpene-producing species such as mint, and for producing monoterpenes in species which typically do not produce monoterpenes such as maize. The invention also provides methods for creating or enhancing  
5 resistance to insects in plants by transforming plants with nucleotide sequences encoding monoterpene synthesis pathway enzymes and generating transformed plants which produce effective amounts of desired monoterpenes in the plant. In this aspect, by "effective amount" is intended that amount of a monoterpene, alone or in combination with other agents, that can effect a reduction, amelioration,  
10 prevention, or elimination of a plant-insect interaction.

Thus, the methods of the invention are directed at transforming plant cells with at least one nucleotide sequence encoding a monoterpene synthesis pathway enzyme selected from GPP synthase, limonene synthase, carveol synthase, S-linalool synthase, or combinations thereof.

15 By "monoterpene synthase" is intended an enzyme which catalyzes a reaction having at least one monoterpene as the product of the reaction. More specifically, monoterpene synthases utilized in the methods of the invention include, but are not limited to limonene synthase, carveol synthase (limonene 6-hydroxylase) and S-linalool synthase.

20 By "transgenic plant" is meant any plant or plant cell that has become transformed by the introduction, stable and heritable incorporation, into the subject plant or plant cell, of either native DNA that is under the control of a promoter other than the promoter that typically drives expression of that DNA in a wild-type plant, and that has been introduced back into its host plant, or foreign DNA, i.e.  
25 DNA encoding for a protein not normally found within that plant species.

"Plantlet" refers to a plant sufficiently developed to have a shoot and a root that is asexually reproduced by cell culture.

"Explant" refers to a section or piece of tissue from any part of a plant for culturing.

30 The term "callus" and its plural "calli", refer to an unorganized group of cells formed in response to cutting, severing, or other injury inflicted on plant tissue. Excised pieces of plant tissue and isolated cells can be induced to form callus under the appropriate culture conditions. Callus can be maintained in

culture for a considerable time by transferring or subculturing parts of the callus to fresh medium at regular intervals. The transfer of callus to liquid medium leads to dispersion of the tissue and the formation of a plant cell suspension culture. Callus can be induced to undergo organized development to form shoots and roots.

5 "Embryoid" refers to a structure similar in appearance to a plant zygotic embryo.

By the term "taxon" herein is meant a unit of botanical classification of genus or lower. It thus includes genus, species, cultivar, variety, variant, and other minor taxonomic groups that lack a consistent nomenclature.

10 "Somatic hybrid" and "somatic hybridization" refers generally to stable combination of cellular material, be it protoplast/protoplast or protoplast/cytoplasm combinations, and includes cybrids and cybridization.

A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as a unit of DNA replication *in vivo*; i.e., capable of replication under its  
15 own control.

As used herein, the term "nucleotide sequence" means a DNA or RNA sequence, and can include a cDNA, or genomic DNA, or synthetic DNA sequence, a structural gene or a fragment thereof, or an mRNA sequence, that encodes an active or functional polypeptide.

20 A "vector" is a replicon, such as a plasmid, phage, or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A DNA "coding sequence" is a DNA sequence which is transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a  
25 start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, procaryotic sequences, cDNA from eucaryotic mRNA, genomic DNA sequences from eucaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A  
30 polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

A "promoter sequence" or a "promoter" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream

(3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bound at its 3' terminus by the translation start codon (ATG) of a coding sequence and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription. Within the  
5 promoter sequence will be found a transcription initiation site, as well as protein binding domains responsible for the binding of RNA polymerase. Eucaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes.

DNA "control sequences" refers collectively to promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination  
10 sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in a host cell.

A coding sequence is "operably linked to" or "under the control of" control sequences in a cell when RNA polymerase will bind the promoter sequence and  
15 transcribe the mRNA, which is then translated into the polypeptide encoded by the coding sequence.

A "host cell" is a cell which has been transformed, or is capable of undergoing transformation, by an exogenous DNA sequence.

A cell has been "transformed" by endogenous or exogenous DNA when  
20 such DNA has been introduced inside the cell membrane. The DNA may or may not be integrated into (covalently linked to) chromosomal DNA making up the genome of the transformed cell. In procaryotes, for example, the DNA may be maintained on an episomal element, such as a plasmid. With respect to eucaryotic cells, a stably transformed cell is one in which the DNA has become integrated into  
25 the chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eucaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the DNA.

A "clone" is a population of cells derived from a single cell or common  
30 ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

A "heterologous" region of a DNA construct is an identifiable segment of DNA within or attached to another DNA molecule that is not found in association

with the other molecule in nature. Thus, when the heterologous region encodes a bacterial gene, the gene will usually be flanked by DNA that does not flank the bacterial gene in the genome of the source bacterium. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not  
5 found in nature (e.g., synthetic sequences having codons different from the native gene). "Heterologous" DNA also refers to DNA not found within the host cell in nature. Allelic variation or naturally occurring mutational events do not give rise to a heterologous region of DNA, as these terms are used herein. "Native", "autologous" or "endogenous" DNA, as used herein, refer to DNA that is typically  
10 present in the host in nature.

The term "polypeptide" as used herein is used in its broadest sense, i.e., any polymer of amino acids (dipeptide or greater) linked through peptide bonds. Thus, the term "polypeptide" includes proteins, oligopeptides, protein fragments, analogues, muteins, fusion proteins and the like. The term also encompasses  
15 amino acid polymers as described above that include additional non-amino acid moieties. Thus, the term "polypeptide" includes glycoproteins, lipoproteins, phosphoproteins, metalloproteins, nucleoproteins, as well as other conjugated proteins. The term "polypeptide" contemplates polypeptides as defined above that are recombinantly produced, isolated from an appropriate source, or synthesized.

20 By the term "vector" herein is meant a DNA sequence which is able to replicate and express a foreign gene in a host cell. Typically, the vector has one or more endonuclease recognition sites which may be cut in a predictable fashion by use of the appropriate enzyme. Such vectors are preferably constructed to include additional structural gene sequences imparting antibiotic or herbicide resistance,  
25 which then serve as selectable markers to identify and separate transformed cells. Preferred selection agents include kanamycin, chlorosulfuron, phosphinothricin, hygromycin and methotrexate, and preferred markers are genes conferring resistance to these compounds. A cell in which the foreign genetic material in a vector is functionally expressed has been "transformed" by the vector and is  
30 referred to as a "transformant." A particularly preferred vector is a plasmid, by which is meant a circular double-stranded DNA molecule that is not a part of the chromosomes of the cell.

In carrying out this invention, it will be appreciated that numerous plant expression cassettes and vectors are well known in the art. By the term "expression cassette" is meant a complete set of control sequences including initiation, promoter and termination sequences which function in a plant cell when they flank a structural gene in the proper reading frame. Expression cassettes frequently and preferably contain an assortment of restriction sites suitable for cleavage and insertion of any desired structural gene. It is important that the cloned gene have a start codon in the correct reading frame for the structural sequence. In addition, the plant expression cassette preferably includes a strong promoter sequence at one end to cause the gene to be transcribed at a high frequency, and a poly-A recognition sequence at the other end for proper processing and transport of the messenger RNA. An example of such a preferred (empty) expression cassette into which the DNA sequence of the present invention can be inserted is the pPHI414 plasmid developed by Beach *et al.* of Pioneer Hi-Bred International, Inc., Johnston, IA. Highly preferred plant expression cassettes are designed to include one or more selectable marker genes, such as kanamycin resistance or herbicide tolerance genes.

Methods of the invention include those for manipulating a metabolic pathway in a plant cell by transforming with nucleotide sequences for native limonene-, GPP-, carveol- and S-linalool synthase genes, by transforming with nucleotide sequences encoding amino acid sequences for the respective proteins encoded thereby, as well as fragments and variants thereof. Such native sequences are set forth in SEQ ID NOs: 1-8. SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 and SEQ ID NO:7 set forth the nucleotide sequences for limonene-, GPP-, carveol-, and S-linalool synthase respectively; the corresponding amino acid sequences set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:8 respectively. The methods encompass using the sequences or corresponding antisense sequences in modulating the expression of enzymes involved in monoterpene synthesis, as well as production of monoterpenes in a plant or plant cell. That is, the coding sequences are used to increase the expression of an enzyme while antisense sequences are used to decrease expression. In this aspect, blocking the expression of an enzyme within a pathway by antisense sequences can

be used to accumulate the substrates of that enzyme or to drive the pathway to another end product.

It is recognized that the methods of the invention could be used to manipulate metabolic pathways involving reactions downstream of those catalyzed by GPP- and a monoterpene synthase such as limonene-, carveol- and/or S-linalool synthase as described herein; by transforming plants with nucleotide sequences encoding GPP synthase and at least one monoterpene synthase, or antisense sequences thereof. In this aspect, the methods of the invention encompass manipulating the pathway involving production of the compounds including but not limited to (-)-carvone, (-)-trans-isopiperitol and (-)-trans-isopiperitinone.

Antisense RNA or DNA can be utilized for the accumulation of a particular monoterpene. Alternatively, homologous plant sequences or partial plant sequences can be used. For example, an antisense carveol synthase sequence can be used to cause the accumulation of limonene. In this manner, the metabolic pathway of interest can be manipulated for the high production of any particular monoterpene of interest in the pathway.

Likewise, the pathway can be manipulated to decrease levels of a particular compound by transformation of antisense sequences which prevent the conversion of the precursor compound into the particular compound being regulated. For example, conversion of GPP to S-linalool can be blocked by antisense sequences to S-linalool synthase.

In the same manner, to increase the biosynthesis of a particular desired monoterpene, antisense constructs can be used to block the conversion of a common substrate to one monoterpene, thereby shunting the common substrate to the pathway for the desired monoterpene, while additionally blocking downstream conversion of the desired monoterpene to a further downstream metabolite. For example, an antisense sequence to S-linalool synthase can be used to shunt the common substrate GPP to the pathway for limonene biosynthesis, while additionally antisense sequence to carveol synthase can be used to block conversion of limonene to carveol.

Any means for producing a plant comprising GPP- and at least one monoterpene synthase coding sequence are encompassed by the methods of the present invention. For example, the second (or additional) gene of interest can be

used to transform a plant at the same time as the GPP synthase gene (cotransformation); the second gene can be introduced into a plant that has already been transformed with the GPP synthase gene; GPP synthase can be transformed into a plant has already been transformed with the second gene; or alternatively, transformed plants, one expressing the GPP synthase and one expressing the second gene, can be crossed to bring the genes together in the same plant. Subsequent crosses or transformations can bring additional sequences together in the plant.

The use of fragments and variants of the nucleotide and amino acid sequences are encompassed within the scope of the invention. By "fragment" is intended a portion of the nucleotide sequence or a portion of the amino acid sequence and hence protein encoded thereby. Fragments of a nucleotide sequence may encode protein fragments that retain the biological activity of the native protein. Alternatively, fragments of a nucleotide sequence that are useful as hybridization probes generally do not encode fragment proteins retaining biological activity. Thus, fragments of a nucleotide sequence may range from at least about 15 nucleotides, about 50 nucleotides, about 100 nucleotides, and up to the entire nucleotide sequence encoding the monoterpene synthase proteins utilized in the methods of the invention. Fragments of the invention include antisense sequences used to decrease expression of the monoterpene synthase genes utilized in the methods of the invention. Such antisense fragments may vary in length ranging from at least about 15 nucleotides, about 50 nucleotides, about 100 nucleotides, up to and including the entire coding sequence.

By "variants" is intended substantially similar sequences. For nucleotide sequences, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of the GPP- and carveol synthase genes. Generally, nucleotide sequence variants of the invention will have at least 70%, generally, 80%, preferably up to 90-95% sequence identity to the native nucleotide sequence.

By "variant" protein is intended a protein derived from the native protein by deletion (so-called truncation) or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native protein; deletion or addition of one or more amino acids at one or more sites in the native protein; or substitution of one

or more amino acids at one or more sites in the native protein. Such variants may result from, for example, genetic polymorphism or from human manipulation. Methods for such manipulations are generally known in the art.

For example, amino acid sequence variants of the polypeptide can be prepared by mutations in the DNA sequence encoding the native protein of interest. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York); Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods Enzymol.* 154:367-382; Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor, New York); U.S. Patent No. 4,873,192; and the references cited therein; herein incorporated by reference. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.* (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be preferred.

In constructing variants of the limonene-, GPP-, carveol and S-linalool synthase proteins of interest, modifications to the nucleotide sequences encoding the variants will be made such that variants continue to possess the desired activity. Obviously, any mutations made in the DNA encoding the variant protein must not place the sequence out of reading frame and preferably will not create complementary regions that could produce excessive secondary mRNA structure. See EP Patent Application Publication No. 75,444.

Thus, nucleotide sequences utilized in the methods of the invention and the proteins encoded thereby include the native forms as well as variants thereof. The variant proteins will be substantially homologous and functionally equivalent to the native proteins. A variant of a native protein is "substantially homologous" to the native protein when at least about 80-85%, more preferably at least about 90%, and most preferably at least about 95% of its amino acid sequence is identical to the amino acid sequence of the native protein. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system.

Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Maniatis *et al.* (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York; Brown, T.A. *Gene Cloning: An Introduction* (2nd Ed.) Chapman & Hall, London (1990).

5           Alternatively, sequence identity can be determined utilizing a number of available computer programs including but not limited to CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from  
10   Genetics Computer Group (GCG), 575 Science Drive, Madison, Wisconsin, USA). Alignments using these programs can be performed using the default parameters. A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. BLAST nucleotide searches can be performed with the BLASTN program, score =  
15   100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the invention. BLAST protein searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a protein or polypeptide of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST  
20   2.0) can be utilized. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used. See <http://www.ncbi.nlm.nih.gov>. Alignment may also be  
25   performed manually by inspection. For purposes of the present invention, comparison of nucleotide or protein sequences for determination of percent sequence identity to the sequences described herein is preferably made using the Gapped BLAST program (Version 2.0 or later) with its default parameters or any equivalent program. By "equivalent program" is intended any sequence  
30   comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by the preferred program.

By "functionally equivalent" is intended that the sequence of the variant defines a chain that produces a protein having substantially the same biological effect as the native protein of interest. Such functionally equivalent variants that comprise substantial sequence variations are also encompassed by the invention.

5 Thus, for purposes of the present invention, a functionally equivalent variant of GPP synthase will catalyze the formation of GPP from dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP); that of limonene synthase will catalyze the formation of limonene from GPP; that of carveol synthase will catalyze the formation of carveol from limonene; and that of S-linalool synthase

10 will catalyze the formation of S-linalool from GPP.

In view of the ability to transform crop plants to express various heterologous compounds, it would be desirable to transform maize plants to express monoterpene pathway enzymes and thereby produce effective amounts of the monoterpenes limonene, carveol and/or S-linalool so that by consuming the

15 tissues of the plant an insect, such as larvae of Corn Rootworm, would consume insecticidally effective amounts of the monoterpenes; or be caused to avoid feeding on the plant.

While most gene products are peptides, a monoterpene is not a peptide or peptide derivative and is not expressed from genes in the form of a peptide or peptide derivative, but is produced enzymatically as a secondary metabolite within

20 the cells of some plants. It is determined that the biosynthetic apparatus necessary for the production of the monoterpene limonene may not be present in many plant cells which do not produce limonene, or may not produce detectable, insecticidally effective amounts of limonene, and this appears to include maize cells. Such plant

25 cells must be engineered with at least one enzyme which can be produced through the expression of exogenous (heterologous) genes. One such enzyme is limonene synthase, also known as limonene cyclase, which can directly synthesize limonene from geranyl pyrophosphate (GPP), which is found widely in both procaryotic and eucaryotic cells, although, as discussed below, is in some cases not produced

30 in quantities sufficient to make insecticidally effective amounts of limonene.

Since genes which code for a monoterpene synthase can be synthesized, either directly using a DNA sequence obtained by working backwards from the known amino acid sequence of a particular monoterpene synthase and preferably

using plant-preferred codons, or by cloning from natural sources of monoterpenes, the resulting sequence can be inserted into an appropriate expression cassette, and introduced into cells of a susceptible plant species or a suitable endophytic bacterium, so that an especially preferred embodiment of this method involves  
5 inserting into the genome of the plant or bacterium a DNA sequence coding for a monoterpene synthase, in proper reading frame relative to transcription initiator and promoter sequences active in the plant or bacterium. Transcription and translation of the coding sequence under control of the regulatory sequences, can cause expression of the enzyme at levels which provide an effective amount of a  
10 monoterpene such as limonene in the tissue of the plant which are normally infested by the larvae.

As an illustration, it can be noted that Colby *et al.*, at the Keystone Symposium on Crop Improvement via Biotechnology: An International Perspective, Keystone, Colorado, USA, April 10-16, 1992, as reported in *J. Cell*  
15 *Biochem. Suppl.* 16 F, 230 (1992), have isolated and characterized cDNA encoding limonene cyclase from spearmint. To isolate and study the gene(s) (sic) encoding limonene synthase and to produce enough of the enzyme for structural studies, they used standard methods to extract RNA from young leaves of *Mentha spicata* and constructed a cDNA library in  $\lambda$  ZAP XR (Stratagene) from poly (A) + RNA.  
20 They designed three degenerate oligonucleotides based on internal amino acid sequences obtained from Edman degradation of purified limonene synthase and screened 250,000 clones to identify six positive clones that hybridized to all three oligonucleotides. The resulting clones could be used in the methods of this invention which involve plant transformation. However, Colby *et al.* indicate no  
25 appreciation of the value of the enzyme in conferring resistance to insects in plants.

In certain plants, including maize, at least one additional gene encoding GPP synthase is required for generation of plants with resistance to insects. Due to the fact that natural levels of GPP are low in such plants, there may be inadequate amounts of GPP for limonene production in these plants when they are  
30 transformed solely with the limonene synthase gene. In contrast, GPP levels in other plant species, such as spearmint, is adequate for limonene production. In such species, some GPP is used to generate limonene, some to generate other metabolites. Because GPP is derived from a pathway that is common among plant

species, the introduction of the GPP synthase gene and at least one monoterpene synthase gene such as limonene synthase into plant species lacking GPP can generate transgenic plants capable of producing GPP, and a desired monoterpene at levels effective to confer resistance to insects.

5 In this manner, the invention encompasses transforming plants with nucleotide sequences encoding GPP synthase and limonene synthase for the generation of plants producing effective amounts of limonene; with nucleotide sequences encoding GPP synthase, limonene synthase and carveol synthase for the generation of plants producing effective amounts of carveol; and with nucleotide  
10 sequences encoding GPP synthase and S-linalool synthase for the generation of plants producing effective amounts of S-linalool.

In one embodiment, the plant which can be benefitted by this invention is preferably a plant susceptible to infestation and damage by the larvae of the genus *Diabrotica* or whose harvested material is subject to attack by larvae of that insect.  
15 A prime example is corn (*Zea mays*). Thus the methods of this invention are readily applicable via conventional techniques to numerous plant species, particularly those susceptible to *Diabrotica spp.*, including, without limitation, species from the genera *Fragaria*, *Lotus*, *Medicago*, *Onobrychis*, *Trifolium*, *Trigonella*, *Vigna*, *Citrus*, *Linum*, *Geranium*, *Manicot*, *Daucus*, *Arabidopsis*,  
20 *Brassica*, *Raphanus*, *Sinapis*, *Atropa*, *Capsicum*, *Datura*, *Hyoscyamus*, *Lycopersicon*, *Nicotiana*, *Solanum*, *Petunia*, *Digitalis*, *Majorana*, *Cichorium*, *Helianthus*, *Lactuca*, *Bromus*, *Asparagus*, *Antirrhinum*, *Hemerocallis*, *Nemesia*, *Pelargonium*, *Panicum*, *Pennisetum*, *Ranunculus*, *Senecio*, *Salpiglossis*, *Cucumis*, *Browallia*, *Glycine*, *Lolium*, *Triticum*, and *Datura*.

25 Thus, the methods of the invention are useful to transform plants and create or enhance resistance to insects in plants. By resistance to insects is intended that the plant-insect interaction, preferably a plant-insect pest interaction, is reduced, ameliorated, prevented, or eliminated.

Insect pests include but are not limited to insects selected from the orders  
30 Coleoptera, Diptera, Hymenoptera, Lepidoptera, Mallophaga, Homoptera, Hemiptera, Orthoptera, Thysanoptera, Dermaptera, Isoptera, Anoplura, Siphonaptera, Trichoptera, etc., particularly Coleoptera and Lepidoptera. Insect pests of the invention for the major crops include: Maize: *Ostrinia nubilalis*,

- European corn borer; *Agrotis ipsilon*, black cutworm; *Helicoverpa zea*, corn earworm; *Spodoptera frugiperda*, fall armyworm; *Diatraea grandiosella*, southwestern corn borer; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Diatraea saccharalis*, sugarcane borer; *Diabrotica virgifera*, western corn
- 5 rootworm; *Diabrotica longicornis barberi*, northern corn rootworm; *Diabrotica undecimpunctata howardi*, southern corn rootworm; *Melanotus spp.*, wireworms; *Cyclocephala borealis*, northern masked chafer (white grub); *Cyclocephala immaculata*, southern masked chafer (white grub); *Popillia japonica*, Japanese beetle; *Chaetocnema pulicaria*, corn flea beetle; *Sphenophorus maidis*, maize
- 10 billbug; *Rhopalosiphum maidis*, corn leaf aphid; *Anuraphis maidiradicis*, corn root aphid; *Blissus leucopterus leucopterus*, chinch bug; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus sanguinipes*, migratory grasshopper; *Hylemya platura*, seedcorn maggot; *Agromyza parvicornis*, corn blot leafminer; *Anaphothrips obscurus*, grass thrips; *Solenopsis milesta*, thief ant; *Tetranychus*
- 15 *urticae*, twospotted spider mite; Sorghum: *Chilo partellus*, sorghum borer; *Spodoptera frugiperda*, fall armyworm; *Helicoverpa zea*, corn earworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Feltia subterranea*, granulate cutworm; *Phyllophaga crinita*, white grub; *Eleodes*, *Conoderus*, and *Aeolus spp.*, wireworms; *Oulema melanopus*, cereal leaf beetle; *Chaetocnema pulicaria*, corn
- 20 flea beetle; *Sphenophorus maidis*, maize billbug; *Rhopalosiphum maidis*, corn leaf aphid; *Sipha flava*, yellow sugarcane aphid; *Blissus leucopterus leucopterus*, chinch bug; *Contarinia sorghicola*, sorghum midge; *Tetranychus cinnabarinus*, carmine spider mite; *Tetranychus urticae*, twospotted spider mite; Wheat: *Pseudaletia unipunctata*, army worm; *Spodoptera frugiperda*, fall armyworm;
- 25 *Elasmopalpus lignosellus*, lesser cornstalk borer; *Agrotis orthogonia*, western cutworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Oulema melanopus*, cereal leaf beetle; *Hypera punctata*, clover leaf weevil; *Diabrotica undecimpunctata howardi*, southern corn rootworm; Russian wheat aphid; *Schizaphis graminum*, greenbug; *Macrosiphum avenae*, English grain aphid;
- 30 *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Melanoplus sanguinipes*, migratory grasshopper; *Mayetiola destructor*, Hessian fly; *Sitodiplosis mosellana*, wheat midge; *Meromyza americana*, wheat stem maggot; *Hylemya coarctata*, wheat bulb fly; *Frankliniella*

*fusca*, tobacco thrips; *Cephus cinctus*, wheat stem sawfly; *Aceria tulipae*, wheat curl mite; Sunflower: *Suleima helianthana*, sunflower bud moth; *Homoeosoma electellum*, sunflower moth; *zygogramma exclamationis*, sunflower beetle; *Bothyrus gibbosus*, carrot beetle; *Neolasioptera murtfeldtiana*, sunflower seed  
 5 midge; Cotton: *Heliothis virescens*, cotton budworm; *Helicoverpa zea*, cotton bollworm; *Spodoptera exigua*, beet armyworm; *Pectinophora gossypiella*, pink bollworm; *Anthonomus grandis*, boll weevil; *Aphis gossypii*, cotton aphid; *Pseudatomoscelis seriatus*, cotton fleahopper; *Trialeurodes abutilonea*, bandedwinged whitefly; *Lygus lineolaris*, tarnished plant bug; *Melanoplus*  
 10 *femurrubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Thrips tabaci*, onion thrips; *Frankliniella fusca*, tobacco thrips; *Tetranychus cinnabarinus*, carmine spider mite; *Tetranychus urticae*, twospotted spider mite; Rice: *Diatraea saccharalis*, sugarcane borer; *Spodoptera frugiperda*, fall armyworm; *Helicoverpa zea*, corn earworm; *Colaspis brunnea*, grape colaspis;  
 15 *Lissorhoptrus oryzophilus*, rice water weevil; *Sitophilus oryzae*, rice weevil; *Nephotettix nigropictus*, rice leafhopper; *Blissus leucopterus leucopterus*, chinch bug; *Acrosternum hilare*, green stink bug; Soybean: *Pseudoplusia includens*, soybean looper; *Anticarsia gemmatilis*, velvetbean caterpillar; *Plathypena scabra*, green cloverworm; *Ostrinia nubilalis*, European corn borer; *Agrotis ipsilon*, black  
 20 cutworm; *Spodoptera exigua*, beet armyworm; *Heliothis virescens*, cotton budworm; *Helicoverpa zea*, cotton bollworm; *Epilachna varivestis*, Mexican bean beetle; *Myzus persicae*, green peach aphid; *Empoasca fabae*, potato leafhopper; *Acrosternum hilare*, green stink bug; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Hylemya platura*,  
 25 seedcorn maggot; *Sericothrips variabilis*, soybean thrips; *Thrips tabaci*, onion thrips; *Tetranychus turkestanii*, strawberry spider mite; *Tetranychus urticae*, twospotted spider mite; Barley: *Ostrinia nubilalis*, European corn borer; *Agrotis ipsilon*, black cutworm; *Schizaphis graminum*, greenbug; *Blissus leucopterus leucopterus*, chinch bug; *Acrosternum hilare*, green stink bug; *Euschistus servus*,  
 30 brown stink bug; *Delia platura*, seedcorn maggot; *Mayetiola destructor*, Hessian fly; *Petrobia latens*, brown wheat mite; Oil Seed Rape: *Brevicoryne brassicae*, cabbage aphid; *Phyllotreta cruciferae*, Flea beetle; *Mamestra configurata*, Bertha armyworm; *Plutella xylostella*, Diamond-back moth; *Delia* ssp., Root maggots.

Preferred plants that are to be transformed according to the methods of this invention are cereal crops, including maize, rye, barley, wheat, sorghum, oats, millet, rice, triticale, sunflower, alfalfa, rapeseed and soybean, fiber crops, such as cotton, fruit crops, such as melons, and vegetable crops, including onion, pepper, tomato, cucumber, squash, carrot, crucifer (cabbage, broccoli, cauliflower), eggplant, spinach, potato and lettuce.

While compounds other than monoterpenes have some effective insecticidal activity at high concentrations in pure form, plant cell expression at such high concentrations is either not possible in a living plant cell system, or is not feasible if the commercially useful characteristics of the plant are to be preserved in terms of production of oils, starches, fibers, or other materials. Monoterpenes, on the other hand, are not directly expressed as the gene product, and the peptide or peptides which is or are expressed in the methods of this invention is an enzyme which can catalyze the synthesis of large amounts of a monoterpene(s) in the tissues of the transformed plant (e.g. limonene synthase), and in instances in which it is required, an enzyme which can catalyze the synthesis of large amounts of substrate for a downstream monoterpene synthase (e.g. GPP synthase).

The genes utilized in the invention, including GPP-, carveol, limonene-, and S-linalool synthase genes can be optimized for enhanced expression in plants of interest. See, for example, EPA0359472; WO91/16432; Perlak *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:3324-3328; and Murray *et al.* (1989) *Nucleic Acids Res.* 17:477-498. In this manner, the genes can be synthesized utilizing plant-preferred condons. See, for example, Murray *et al.* (1989) *Nucleic Acids Res.* 17:477-498, the disclosure of which is incorporated herein by reference. In this manner, synthetic genes can also be made based on the distribution of codons a particular host uses for a particular amino acid. Thus, the nucleotide sequences can be optimized for expression in any plant. It is recognized that all or any part of the gene sequence may be optimized or synthetic. That is, synthetic or partially optimized sequences may also be used.

The methods of the invention encompass utilizing naturally occurring nucleotide sequences encoding GPP synthase, and the monoterpene synthases limonene-, carveol- and S-linalool synthases; or utilizing synthetically derived

sequences encoding these proteins. The naturally occurring nucleotide sequences utilized in the methods of the invention are set forth in Figures 6, 8, 10 and 12.

The methods also encompass utilizing nucleotide sequences isolated from various organisms including plants by hybridization with partial sequences  
5 obtained from the natural sequences as set forth above. Conditions that will permit other DNA sequences to hybridize to the DNA sequences set forth herein can be determined in accordance with techniques generally known in the art. For example, hybridization of such sequences may be carried out under conditions of reduced stringency, medium stringency, or high stringency conditions (e.g.,  
10 conditions represented by a wash stringency of 35-40% Formamide with 5x Denhardt's solution, 0.5% SDS, and 1x SSPE at 37°C; conditions represented by a wash stringency of 40-45% Formamide with 5x Denhardt's solution, 0.5% SDS, and 1x SSPE at 42°C; and conditions represented by a wash stringency of 50% Formamide with 5x Denhardt's solution, 0.5% SDS, and 1x SSPE at 42°C,  
15 respectively. See Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). The methods further encompass utilizing nucleotide sequences isolated from various organisms including plants by other well known methods such as PCR using the natural sequences as set forth above.

20 The methods of the invention comprise utilizing expression cassettes with constitutive or tissue-specific promoters. Promoters that may be used in the expression cassettes include without limitation nos, ocs, phaseolin, FMV and other promoters isolated from the DNA of plants or other sources, both natural and synthetic.

25 Constitutive promoters would provide a constant production of the enzymes GPP-, limonene-, carveol and/or S-linalool synthase and thereby the corresponding monoterpene(s). Such constitutive promoters include, for example, the core promoter of the Rsyn7 (U.S. Patent Application Serial No. 08/661,601), the core CaMV 35S promoter (Odell *et al.* (1985) *Nature* 313:810-812); rice actin  
30 (McElroy *et al.* (1990) *Plant Cell* 2:163-171); ubiquitin (Christensen *et al.* (1989) *Plant Mol. Biol.* 12:619-632 and Christensen *et al.* (1992) *Plant Mol. Biol.* 18:675-689); pEMU (Last *et al.* (1991) *Theor. Appl. Genet.* 81:581-588); MAS (Velten *et al.* (1984) *EMBO J.* 3:2723-2730); ALS promoter (U.S. Patent Application Serial

No. 08/409,297), and the like. Other constitutive promoters include, for example, U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; and 5,608,142.

5 An efficient plant promoter that may be used is an overproducing plant promoter. Overproducing plant promoters that may be used in this invention include the promoter of the small sub-unit (ss) of the ribulose-1,5-bisphosphate carboxylase from soybean (Berry-Lowe *et al.* (1982) *J. Mol. and App. Gen.* 1:483-498, and the promoter of the chlorophyll a-b binding protein. However, these two promoters are known to be light-induced in eukaryotic plant cells (see, for  
10 example, *Genetic Engineering of Plants, An Agricultural Perspective*, Cashmore, Pelham, New York, 1983, pp. 29-38, G. Coruzzi *et al.* (1983) *J. Biol. Chem.* 258:1399 and P. Dunsmuir *et al.* (1983) *J. Mol. and App. Gen.* 2:285 and may be less desirable when root expression is desired.

A tissue-specific promoter (or promoters) can be used in any instance  
15 where it may be desirable to localize production of the desired monoterpene to an insect-infested tissue or to a tissue which is efficient in production of a desired enzyme. The utilization of tissue-specific promoters would increase or decrease the expression of monoterpene synthases and production of the corresponding monoterpenes in specific tissues of the plant. It is recognized that in manipulating  
20 the level of monoterpene production as such, it may be desirable to increase or decrease the levels of such molecules in a particular tissue, since tolerance of various tissues to increased expression of specific monoterpenes may vary. Thus, it may be desirable to increase expression in selected tissues, or at varying levels in different tissues by the use of tissue-specific promoters.

25 Particular tissue-specific promoters of interest includes root-preferred promoters. The utilization of such promoters would provide a method of selectively creating or enhancing resistance to insects, and/or manipulating levels of monoterpene molecules in the root. Since corn rootworm attack roots, root-specific promoters are especially preferred for the control of corn rootworm, while  
30 minimizing limonene production in the agronomically valuable parts of the plant. Such selectivity could also be particularly desirable in plants in which the root constitutes the food crop, including, but not limited to carrot, potato, radish, and the like.

Root specific promoters are known and can be selected from the many available from the literature or isolated de novo from various compatible species. For example, Hire, *et al.* (1992) *Plant Mol. Biology*, 20(2): 207-218, describe a root-specific glutamine synthetase gene from soybean. Keller and Baumgartner, (1991) *The Plant Cell*, 3(10):1051-1061, describe a root-specific control element in the GRP 1.8 gene of French bean. Sanger *et al.* (1990) *Plant Mol. Biology*, 14(3): 433-443, discuss the root-specific promoter of the Mannopine Synthase (MAS) gene of *Agrobacterium tumefaciens*. Miao *et al.* (1991) *The Plant Cell*, 3(1):11-22, describe a full-length cDNA clone encoding cytosolic glutamine synthetase (GS), which is expressed in roots and root nodules of soybean. Bogusz *et al.* (1990) *The Plant Cell*, 2(7):633-641, discusses two root-specific promoters isolated from hemoglobin genes from the nitrogen-fixing nonlegume *Parasponia andersonii* and the related non-nitrogen-fixing nonlegume *Trema tomentosa*. The promoters of these genes were linked to a  $\beta$ -glucuronidase reporter gene and introduced into both the nonlegume *Nicotiana tabacum* and the legume *Lotus corniculatus*, and in both instances root-specific promoter activity was preserved. Leach and Aoyagi (1991) *Plant Science (Limerick)* 79(1):69-76, describe their analysis of the promoters of the highly expressed rolC and rolD root-inducing genes of *Agrobacterium rhizogenes*. They concluded that enhancer and tissue-specific DNA determinants are dissociated in those promoters. Teeri *et al.* (1989) *EMBO Journal*, 8(2):343-350, used gene fusion to lacZ to show that the *Agrobacterium* T-DNA gene encoding octopine synthase is especially active in the epidermis of the root tip and that the TR2' gene was root specific in the intact plant and stimulated by wounding in leaf tissue, an especially desirable combination of characteristics for use with an insecticidal or larvicidal gene. The TR1' gene, fused to NPTII, (neomycin phosphotransferase II) showed similar characteristics. Additional root-preferred promoters include the VfENOD-GRP3 gene promoter (Kuster H *et al.* (1995) *Plant Mol. Biol.* 29(4):759-772); and rolB promoter (Capana *et al.* (1994) *Plant Mol. Biol.* 25(4):681-691. See also U.S. Patent Nos. 5,633,363; 5,459,252; 5,401,836; 5,110,732; and 5,023,179.

Other tissue-specific promoters include those described in Yamamoto *et al.* (1997) *Plant J.* 12(2):255-265; Kawamata *et al.* (1997) *Plant Cell Physiol.* 38(7):792-803; Hansen *et al.* (1997) *Mol. Gen. Genet.* 254(3):337-343; Russell *et*

*al.* (1997) *Transgenic Res.* 6(2):157-168; Rinehart *et al.* (1996) *Plant Physiol.* 112(3):1331-1341; Van Camp *et al.* (1996) *Plant Physiol.* 112(2):525-535; Canevascini *et al.* (1996) *Plant Physiol.* 112(2):513-524; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35(5):773-778; Lam (1994) *Results Probl. Cell Differ.* 20:181-196; Orozco *et al.* (1993) *Plant Mol. Biol.* 23(6):1129-1138; Matsuoka *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90(20):9586-9590; and Guevara-Garcia *et al.* (1993) *Plant J.* 4(3):495-505.

Seed-preferred promoters includes both seed-specific promoters (those promoters active during seed development) as well as seed-germinating promoters (those promoters active during seed germination). Such promoters include Cim1 (cytokinin-induced message); cZ19B1 (maize 19KDa zein); milps (myo-inositol-1-phosphate synthase); celA (cellulose synthase); end1 (*Hordeum vertgase* mRNA clone END1); and imp3 (myo-inositol monophosphate-3). For dicots, particular promoters include phaseolin, napin,  $\beta$ -conglycinin, soybean lectin, and the like. For monocots, particular promoters include maize 15Kd zein, 22KD zein, 27kD zein, waxy, shrunken 1, shrunken 2, globulin 1, etc.

In native tissues which synthesize monoterpenes, GPP-, limonene- and S-linalool synthases are targeted to plastids via plastid targeting sequences (transit peptides) typically contained at the N-terminus of the preprotein forms of the enzymes. The targeting sequences are then cleaved to release the mature enzymes in plastids. Such transit peptides can be identified in the primary amino acid sequences of the preproteins by those ordinarily skilled in the art. For example, see Colby *et al.* (1993) *J. Biol. Chem.* 268(31):23016-23024, for the transit peptide sequence of limonene synthase. Native carveol synthase is associated with the endoplasmic reticulum, while native carveol dehydrogenase could be cytoplasmic.

Furthermore, the substrates of GPP synthase, IPP (IDP) and DMAPP (DMADP) can be found in plastids or in the cytoplasm (Bohlmann *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95:4126-4133). In plastids, these substrates are most likely synthesized via the glyceraldehyde-3-phosphate/pyruvate (Rohmer) pathway, and in the cytosol via the mevalonate pathway in the cytosol or the mevalonate pathway.

Thus, for manipulating a metabolic pathway involving monoterpene production, it would be beneficial to target GPP-, limonene-, and S-linalool

synthase to plastids; and carveol synthase and dehydrogenase to the endoplasmic reticulum (ER). This targeting could be achieved by use of the native targeting sequences contained in the sequences of the native proteins, or by addition or exchange of heterologous subcellular targeting signals. Alternatively, the enzymes  
5 utilized in the methods of the invention could be directed to the cytoplasm by deletion of the plastid and/or ER targeting signals. Methods for deletion, exchange and addition of nucleotide sequences are well known in the art, and can be readily used for manipulation of nucleotide segments encoding targeting signals of interest as described herein.

10 Heterologous sequences which can be used to target the desired enzymes of the invention to plastids include chloroplast targeting sequences. Chloroplast targeting sequences are known in the art and include the chloroplast small subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco), (de Castro Silva Filho *et al.* (1996) *Plant Mol. Biol.* 30:769-780; Schnell, *et al.* (1991) *J. Biol. Chem.* 266(5):3335-3342); 5-(enolpyruvyl)shikimate-3-phosphate synthase (EPSPS) (Archer *et al.* (1990) *J. Bioenerg. Biomemb.* 22(6):789-810); tryptophan synthase (Zhao *et al.* (1995) *J. Biol. Chem.* 270(11):6081-6087); plastocyanin (Lawrence *et al.* (1997) *J. Biol. Chem.* 272(33):20357-20363), chorismate synthase (Schmidt *et al.* (1993) *J. Biol. Chem.* 268(36):27477-27457), and the light harvesting  
15 20 chlorophyll a/b binding protein (LHBP) (Lamppa *et al.* (1988) *J. Biol. Chem.* 263:14996-14999). See also Von Heijne *et al.* (1991) *Plant Mol. Biol. Rep.* 9:104-126; Clark *et al.* (1989) *J. Biol. Chem.* 264:17544-17550; della-Cioppa *et al.* (1987) *Plant Physiol.* 84:965-968; Romer *et al.* (1993) *Biochem. Biophys. Res Commun.* 196:1414-1421; and Shah *et al.* (1986) *Science* 233:478-481.

25 Likewise, methods for transformation of chloroplasts are known in the art. See, for example, Svab *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:8526-8530; Svab and Maliga (1993) *Proc. Natl. Acad. Sci. USA* 90:913-917; Staub and Maliga (1993) *Embo J.* 12 601-606. The method relies on particle gun delivery of DNA containing a selectable marker and targeting of the DNA to the plastid genome  
30 through homologous recombination. Additionally, plastid transformation can be accomplished by transactivation of a silent plastid-borne transgene by tissue-specific expression of a nuclear-encoded and plastid-directed RNA polymerase.

Such a system has been reported in McBride *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:7301-7305.

The nucleotide sequences utilized in methods of the invention can be introduced into any plant. The sequences to be introduced may be used in  
5 expression cassettes for expression in any plant of interest where expression in the plant is necessary for transcription.

While it may be preferable to express the sequences using heterologous promoters, the native promoter sequences may be used. Such constructs would change expression levels of the enzymes of the invention in the plant or plant cell.  
10 Thus, the phenotype of the plant or plant cell is altered.

Where expression cassettes are needed, such expression cassettes will comprise a transcriptional initiation region linked to the coding sequence or antisense sequence of the nucleotide of interest. Such an expression cassette is provided with a plurality of restriction sites for insertion of the sequence to be  
15 under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

The transcriptional initiation region, the promoter, may be native or analogous or foreign or heterologous to the plant host. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. By foreign is  
20 intended that the transcriptional initiation region is not found in the native plant into which the transcriptional initiation region is introduced. As used herein a chimeric gene comprises a coding sequence operably linked to a transcription initiation region that is heterologous to the coding sequence.

The transcriptional cassette will include in the 5'-to-3' direction of  
25 transcription, a transcriptional and translational initiation region, a DNA sequence of interest, and a transcriptional and translational termination region functional in plants. The termination region may be native with the transcriptional initiation region, may be native with the DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid  
30 of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also Guerineau *et al.* (1991) *Mol. Gen. Genet.* 262:141-144; Proudfoot (1991) *Cell* 64:671-674; Sanfacon *et al.* (1991) *Genes Dev.* 5:141-149; Mogen *et al.* (1990) *Plant Cell.* 2:1261-1272; Munroe *et al.* (1990) *Gene* 91:151-

158; Ballas *et al.* (1989) *Nucleic Acids Res.* 17:7891-7903; Joshi *et al.* (1987) *Nucleic Acids Res.* 15:9627-9639.

The methods of the invention encompass use of expression cassettes for expression of nucleotide sequences encoding GPP- and monoterpene synthases in the plant of interest. The cassette will include 5' and 3' regulatory sequences operably linked to the sequence of interest. The cassette may additionally contain at least one additional sequence to be cotransformed into the organism. Alternatively, the additional sequence(s) can be provided on another expression cassette.

10       Where appropriate, sequences utilized in the methods of the invention and additional gene(s) may be optimized for increased expression in the transformed plant. That is, these nucleotide sequences can be synthesized using plant preferred codons for improved expression. Methods are available in the art for synthesizing plant preferred genes. See, for example, U.S. Patent Nos. 5,380,831, 5,436, 391, 15       and Murray *et al.* (1989) *Nucleic Acids Res.* 17:477-498, herein incorporated by reference.

Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, 20       and other such well-characterized sequences which may be deleterious to gene expression. The G-C content of the sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures.

25       The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein *et al.* (1989) *Proc. Nat. Acad. Sci. USA* 86:6126-6130); potyvirus 30       leaders, for example, TEV leader (Tobacco Etch Virus) (Allison *et al.* (1986)); MDMV leader (Maize Dwarf Mosaic Virus) (*Virology* 154:9-20); human immunoglobulin heavy-chain binding protein (BiP) (Macejak and Sarnow (1991) *Nature* 353:90-94); untranslated leader from the coat protein mRNA of alfalfa

mosaic virus (AMV RNA 4) (Jobling and Gehrke (1987) *Nature* 325:622-625); tobacco mosaic virus leader (TMV) (Gallie *et al.* (1989) *Molecular Biology of RNA*, pages 237-256); and maize chlorotic mottle virus leader (MCMV) (Lommel *et al.* (1991) *Virology* 81:382-385). See also Della-Cioppa *et al.* (1987) *Plant Physiology* 84:965-968. Other methods known to enhance translation can also be utilized, for example, introns, and the like.

In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, *e.g.*, transitions and transversions, may be involved.

The methods of the invention can be used in transforming or transfecting any plant. In this manner, genetically modified plants, plant cells, plant tissue, seed, and the like can be obtained. Transformation protocols as well as protocols for introducing nucleotide sequences into plants may vary depending on the type of plant or plant cell, *i.e.* monocot or dicot, targeted for transformation. Suitable methods of introducing nucleotide sequences into plant cells and subsequent insertion into the plant genome include microinjection (Crossway *et al.* (1986) *Biotechniques* 4:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606, *Agrobacterium*-mediated transformation (Hinchee *et al.* (1988) *Biotechnology* 6:915-921), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J.* 3:2717-2722), and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent No. 4,945,050; Tomes *et al.* (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); and McCabe *et al.* (1988) *Biotechnology* 6:923-926). Also see Weissinger *et al.* (1988) *Annual Rev. Genet.* 22:421-477; Sanford *et al.* (1987) *Particulate Science and Technology* 5:27-37 (onion); Christou *et al.* (1988) *Plant Physiol.* 87:671-674 (soybean); McCabe *et al.* (1988) *Bio/Technology* 6:923-926 (soybean); Finer and McMullen (1991) *In Vitro Cell Dev. Biol.*

- 27P:175-182 (soybean); Singh *et al.* (1998) *Theor. Appl. Genet.* 96:319-324 (soybean); Datta *et al.* (1990) *Biotechnology* 8:736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology* 6:559-563 (maize); Tomes *et al.* "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg (Springer-Verlag, Berlin) (1995) (maize); Klein *et al.* (1988) *Plant Physiol.* 91:440-444 (maize); Fromm *et al.* (1990) *Biotechnology* 8:833-839 (maize); Hooydaas-Van Slogteren and Hooykaas (1984) *Nature (London)* 311:763-764; Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:5345-5349 (Liliaceae); De Wet *et al.* (1985) in *The Experimental Manipulation of Ovule Tissues*, ed. Chapman *et al.* (Longman, New York), pp. 197-209 (pollen); Kaeppler *et al.* (1990) *Plant Cell Reports* 9:415-418; and Kaeppler *et al.* (1992) *Theor. Appl. Genet.* 84:560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell* 4:1495-1505 (electroporation); Li *et al.* (1993) *Plant Cell Reports* 12:250-255 and Christou and Ford (1995) *Annals of Botany* 75:407-413 (rice); Osjoda *et al.* (1996) *Nature Biotechnology* 14:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference. Thus, the invention provides a method for imparting insect resistance in *Agrobacterium tumefaciens*-susceptible plants in which the expression cassette is introduced into the cells by infecting the cells with *Agrobacterium tumefaciens*, a plasmid of which has been modified to include a plant expression cassette which expresses GPP- and/or a monoterpene synthase in the manner of this invention.

The modified plant may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell. Reports* 5:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting plants or hybrid plants the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that the subject phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure the desired phenotype or other property has been achieved.

In a preferred embodiment, the invention provides methods of imparting resistance to *Diabrotica spp.* to plants of a susceptible taxon, comprising the steps of:

- (a) culturing cells or tissues from at least one plant from the taxon,
- 5 (b) introducing into the cells of the cell or tissue culture at least one copy of an expression cassette comprising a structural gene coding for at least one monoterpene synthase or both a monoterpene synthase and GPP synthase, operably linked to a promoter that drives expression in a plant cell, and
- (c) regenerating whole plants from the cell or tissue culture with
- 10 resistance to insects. Once whole plants have been obtained, they can be sexually or clonally reproduced in such manner that at least one copy of the sequence provided by the expression cassette is present in the cells of progeny of the reproduction.

Alternatively, once a single transformed plant has been obtained by the

15 foregoing recombinant DNA method, conventional plant breeding methods can be used to transfer the structural gene or genes and associated regulatory sequences via crossing and backcrossing. Such intermediate methods will comprise the further steps of:

- (a) sexually crossing the plant having resistance to insects with a plant
- 20 from the taxon susceptible to insects;
- (b) recovering reproductive material from the progeny of the cross; and
- (c) growing plants having resistance to insects from the reproductive material. Where desirable or necessary, the agronomic characteristics of the susceptible taxon can be substantially preserved by expanding this method to
- 25 include the further steps of repetitively:
  - (a) backcrossing the progeny having resistance to insects with plants from taxon susceptible to insects; and
  - (b) selecting for the expression of resistance to insects (or an associated marker gene) or monoterpene production among the progeny of the backcross,
  - 30 until the desired percentage of the characteristics of the susceptible taxon are present in the progeny along with the gene imparting resistance to insects.

Insect pests of harvested material, including those of stored grain, can also be targets for the methods of this invention. In view of this, the invention also

provides methods for creating or enhancing resistance to insect pests in harvested materials and products obtained from harvested materials, by expressing at least one monoterpene synthase and/or GPP synthase in the plant such that effective amounts of the desired monoterpene is produced in the harvested material and products obtained from such material.

The following examples are offered by way of illustration and not by way of limitation. The following description further exemplifies the compositions of this invention and the methods of making and using them. However, it will be understood that other methods, known by those of ordinary skill in the art to be equivalent, can also be employed.

### EXAMPLES 1-6

#### Insect larvae inhibition and toxicity assays for

#### Southern Corn Rootworm (SCR) and European Corn Borer (ECB)

Bioassay diets were prepared as described in Czapla and Lang in "Effect of Plant Lectins on the Larval Development of European Corn Borer (Lepidoptera: Pyralidae) and Southern Corn Rootworm (Coleoptera: Chrysomelidae)", *J. Econ. Entomol.* 83:2480-85 (1990), except that low melting temperature agarose replaced the regular agarose so that the diets could be chilled to 37°C prior to the addition of limonene (one assay used the regular agarose diet).

Results were as follows. In Examples 1-4, the test larvae were Southern Corn Rootworm. In Examples 5-6, the test larvae were European Corn Borer. The results of each experiment represent the average from 16-32 insects. All limonene concentrations (ppm) are by weight. The SCR data indicate that limonene is effective against the larvae, but when limonene was used in the same protocol against ECB, little or no effect was seen.

$$\% \text{ Corrected Mortality} = 100 \times \frac{(\text{mortality of treated} - \text{control})}{(100 - \text{control})}$$

$$\% \text{ Wt. Reduction} = 100 \times \frac{(\text{control weight} - \text{treated weight})}{(\text{control wt})}$$

Example:	<u>1</u>		<u>2</u>	
Limonene	% Corr.	% weight	% Corr.	% weight
<u>ppm</u>	<u>Mortal.</u>	<u>Reduction</u>	<u>Mortal.</u>	<u>Reduction</u>
10,000	57	64	80	86
1,000	49	52	19	30
100	57	52	15	6

Example:	<u>3</u>		<u>4</u>	
Limonene	% Corr.	% weight	% Corr.	% weight
<u>ppm</u>	<u>Mortal.</u>	<u>Reduction</u>	<u>Mortal.</u>	<u>Reduction</u>
10,000	96	64	44	0
1,000	7	0	22	0
100	26	0	19	0

## Average SCR Results:

Limonene	% Corr.	% weight
<u>ppm</u>	<u>Mortal.</u>	<u>Reduction</u>
10,000	69	54
1,000	24	21
100	29	15

5

Example:	<u>5</u>		<u>6</u>	
Limonene	% Corr.	% weight	% Corr.	% weight
<u>ppm</u>	<u>Mortal.</u>	<u>Reduction</u>	<u>Mortal.</u>	<u>Reduction</u>
10,000	0	28	0	0
1,000	0	16	0	0
100	0	1	0	7

## Average ECB Results:

Limonene	% Corr.	% weight
<u>ppm</u>	<u>Mortal</u>	<u>Reduction</u>
10,000	0	14
1,000	0	8
100	0	4

## EXAMPLE 7

Maize callus cultures were transformed by microprojectile bombardment using plasmids containing a cloned gene coding for the limonene synthase (limonene cyclase) enzyme driven by a ubiquitin promoter and a ubiquitin intron and followed downstream by a PIN-II terminator. Whole, fertile plants were regenerated from the transformed callus and analyzed for limonene synthase and limonene. Representative results from one series were as follows:

10

<u>Clone #</u>	<u>Callus LS<sup>1</sup></u>	<u>Callus</u> <u>Limonene<sup>2</sup></u>	<u>Plant</u>	<u>Plant LS</u>	<u>Plant</u> <u>Limonene</u>
C6	22539	ND	3	1950	ND
			7	2000	ND
C19	9900	ND	6	2100	ND
C3	2550	ND	3	1900	ND
			4	1650	ND

In other experiments, transgenic maize plants, and tissues that exhibited high expression of the limonene synthase protein were produced. The enzyme was extracted from transformed plants and tissues and allowed to react with tritium-labeled geranyl pyrophosphate (GPP). The extracted enzyme converted GPP to limonene, showing that a functional enzyme was being produced. In addition, western blots were done and confirmed the presence of LS protein in transgenic

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1 LS = Limonene Synthase

2 ND = not detected above 0.5 ng/g fresh wt.

tissues but not in negative control tissues. Seed was collected from the transformed plants. In sum, whole fertile, transformed plants have been produced that express active limonene cyclase (synthase) according to the teaching of the above-identified application.

5           Western blots were also performed on chloroplasts isolated from plants grown from T1 and T2 seed which expressed the mint limonene synthase gene (Figure 6) in leaf tissue. Thus, the mint limonene synthase was properly targeted to maize plastids and the protein was processed to the correct size, indicating that the mint limonene synthase plastid targeting sequence can be used to target  
10   proteins to maize plastids.

### EXAMPLE 8

In view of the results set forth in Example 7, demonstrating expression of active limonene synthase in whole, fertile, transformed plants, larvicidally  
15   effective amounts of limonene can be produced in such transgenic plants where sufficient substrate is present for the limonene synthase enzyme to act on. Accordingly, maize callus cultures are transformed by art recognized microprojectile bombardment methods using plasmids containing genes coding for both the limonene synthase enzyme and the GPP synthase enzyme, driven, for  
20   example, by one or more promoters (a ubiquitin promoter, for example) and followed downstream by, for example, a PIN-II terminator. Whole, fertile plants are regenerated from the transformed callus and analyzed for presence of and/or activity of both enzymes, and are also analyzed for the presence of limonene.

Alternatively, callus is generated from transgenic plants that contain and  
25   express the limonene synthase transgene, and such callus cultures are transformed as described above, except that such callus is transformed using plasmids containing a gene coding for the GPP synthase protein. The whole, fertile, transgenic plants regenerated from such transformed callus produce larvicidally effective amounts of limonene.

30

**EXAMPLE 9**

Average minimum levels of various monoterpenes required for 100% mortality of Western corn rootworm were determined by the methods described in Examples 1-6 as follows:

- 5           Limonene-----200 ppm  
          S-linalool---50 ppm  
          carveol-----25 ppm

**EXAMPLE 10: Construction of Expression Vectors**

- 10           Nucleotide sequences encoding limonene, GPP-, carveol and S-linalool synthase, as set forth in SEQ ID NOs: 1, 3, 5, and 7 respectively, are cloned into a plasmid vector, such as that shown in Figure 2, in the sense orientation so that they are under the transcriptional control of the ubiquitin promoter. A selectable marker gene may reside on this plasmid or may be introduced as part of a second plasmid.
- 15           The transformation construct is then available for introduction into maize embryos by bombardment methods as described in Example 12.

**EXAMPLE 11: Construction of Expression Vectors**

- 20           Nucleotide sequences encoding GPP- and S-linalool synthase as described in EXAMPLE 10 were cloned in the sense orientation into an expression vector as shown in in Figure 3(PHP14099); such that both coding sequences are under the transcriptional control of the ubiquitin promoter. The plasmid contains the
- 25           selectable marker gene PAT.

- Nucleotide sequences encoding GPP-, limonene- and carveol synthases as described in EXAMPLE 10 were cloned in the sense orientation into an expression vector as shown in Figure 4(PHP14100); such that all three coding sequences are under the transcriptional control of the ubiquitin promoter. The plasmid contains
- 30           the selectable marker gene PAT.

          The transformation constructs PHP14099 and PHP14100 are available for introduction into maize embryos by bombardment methods as described in Example 12.

**EXAMPLE 12: Transformation and Regeneration of Maize Callus**

Immature maize embryos from greenhouse donor plants are bombarded with a plasmid containing the disclosed GPP- or monoterpene synthase gene operably linked to the ubiquitin promoter plus a plasmid containing the selectable marker gene PAT (Wohlleben et al. (1988) Gene 70:25-37) that confers resistance to the herbicide Bialophos. Transformation is performed as follows. All media recipes are in the Appendix.

10 **Preparation of Target Tissue**

The ears are surface sterilized in 30% Chlorox bleach plus 0.5% Micro detergent for 20 minutes, and rinsed two times with sterile water. The immature embryos are excised and placed embryo axis side down (scutellum side up), 25 embryos per plate. These are cultured on 560 L medium for 4 days prior to bombardment, in the dark. The day of bombardment, the embryos are transferred to 560 Y medium for 4 hours, arranged within the 2.5-cm target zone.

**Preparation of DNA**

A plasmid vector comprising the disclosed GPP- or monoterpene synthase operably linked to the ubiquitin promoter is constructed. This plasmid DNA plus plasmid DNA containing a PAT selectable marker is precipitated onto 1.1  $\mu\text{m}$  (average diameter) tungsten pellets using a  $\text{CaCl}_2$  precipitation procedure as follows:

- 100  $\mu\text{l}$  prepared tungsten particles in water
- 25 10  $\mu\text{l}$  (1  $\mu\text{g}$ ) DNA in TrisEDTA buffer (1  $\mu\text{g}$  total)
- 100  $\mu\text{l}$  2.5 M  $\text{CaCl}_2$
- 10  $\mu\text{l}$  0.1 M spermidine

Each reagent is added sequentially to the tungsten particle suspension, while maintained on the multitube vortexer. The final mixture is sonicated briefly and allowed to incubate under constant vortexing for 10 minutes. After the precipitation period, the tubes are centrifuged briefly, liquid removed, washed with 500 ml 100% ethanol, and centrifuged for 30 seconds. Again the liquid is

removed, and 105  $\mu$ l 100% ethanol is added to the final tungsten particle pellet. For particle gun bombardment, the tungsten/DNA particles are briefly sonicated and 10  $\mu$ l spotted onto the center of each macrocarrier and allowed to dry about 2 minutes before bombardment.

5

#### Particle Gun Treatment

The sample plates are bombarded at level #4 in particle gun #HE34-1 or #HE34-2. All samples receive a single shot at 650 PSI, with a total of ten aliquots taken from each tube of prepared particles/DNA.

10

#### Subsequent Treatment

Following bombardment, the embryos are kept on 560Y medium for 2 days, then transferred to 560R selection medium containing 3 mg/liter Bialophos, and subcultured every 2 weeks. After approximately 10 weeks of selection, selection-resistant callus clones are sampled for PCR and activity of the acetyl-CoA synthetase gene of interest. Positive lines are transferred to 288J medium to initiate plant regeneration. Following somatic embryo maturation (2-4 weeks), well-developed somatic embryos are transferred to medium for germination and transferred to the lighted culture room. Approximately 7-10 days later, developing plantlets are transferred to medium in tubes for 7-10 days until plantlets are well established. Plants are then transferred to inserts in flats (equivalent to 2.5" pot) containing potting soil and grown for 1 week in a growth chamber, subsequently grown an additional 1-2 weeks in the greenhouse, then transferred to classic 600 pots (1.6 gallon) and grown to maturity. Plants are monitored for expression of the GPP-synthase or monoterpene synthase gene of interest.

25

APPENDIX

Ingredient	Amount	Unit
D-I H <sub>2</sub> O	900.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	1.600	g
N6 Macronutrients 10X Stock ##	60.000	ml
Potassium Nitrate	1.680	g
B5H Minor Salts 1000X ###	0.600	ml
B5H Fe Na EDTA 100X ####	6.000	ml
Eriksson's Vitamin Mix (1000X SIGMA-1511)	0.400	ml
S & H Vitamin Mixture 100X Stock (S3766)	6.000	ml
Thiamine.HCL 0.4mg/ml	0.500	ml
L-Proline	1.980	g
Casein Hydrolystate (acid)	0.300	g
Sucrose	20.000	g
Glucose	0.600	g
2,4-D 0.5 mg/ml	1.600	ml
Gelrite @	2.000	g
Dicamba 1 mg/ml #	1.200	ml
Silver Nitrate 2 mg/ml #	1.700	ml

Directions:

@= Add after bringing up to volume

5   #= Add after sterilizing and cooling to temp.

Dissolve ingredients in polished D-I H<sub>2</sub>O in sequence

Adjust to pH 5.8

Bring up to volume with polished D-I H<sub>2</sub>O after adjusting pH

Sterilize and cool to 60°C.

10   ##= Dissolve 1.660 g of Calcium Chloride Dihydrate in 950.000 ml of polished D-I H<sub>2</sub>O. Then dissolve 4.629 of Ammonium Sulfate; 4.000 g of Potassium Phosphate Monobasic KH<sub>2</sub>PO<sub>4</sub>; 1.850 g of Magnesium Sulfate 7-H<sub>2</sub>O, MgSO<sub>4</sub>, 7H<sub>2</sub>O; and 28.300 g of Potassium Nitrate into sequence. Bring up to volume with polished D-I H<sub>2</sub>O.

### = Dissolve 3.000 g of Boric Acid; 10.000 g of Manganous Sulfate Monohydrate; 0.250 g of Sodium Molybdate Dihydrate; and 0.750 g of Potassium Iodide in polished D-I H<sub>2</sub>O in sequence. Bring up to volume with polished D-I H<sub>2</sub>O.

- 5 #### = Dissolve 3.700 g of Disodium EDTA Dihydrate and 2.790 g of Ferrous Sulfate 7-Hydrate into D-I H<sub>2</sub>O. Bring up to volume with D-I H<sub>2</sub>O.

Total Volume (L) = 1.00

## 604 A

Ingredient	Amount	Unit
D-I H <sub>2</sub> O	900.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	1.600	g
N6 Macronutrients 10X Stock ##	60.000	ml
Potassium Nitrate	1.680	g
B5H Minor Salts 1000X ###	0.600	ml
B5H Fe Na EDTA 100X ####	6.000	ml
Eriksson's Vitamin Mix (1000X SIGMA-1511)	0.400	ml
S & H Vitamin Mixture 100X Stock (S3766)	6.000	ml
Thiamine.HCL 0.4mg/ml	0.500	ml
L-Proline	1.980	g
Casein Hydrolysate (acid)	0.300	g
Sucrose	20.000	g
Glucose	0.600	g
2, 4-D 0.5mg/ml	1.600	ml
Gelrite @	2.000	g
Dicamba 1mg/ml #	1.200	ml
Silver Nitrate 2mg/ml #	1.700	ml
Bialaphos 1 mg/ml #	3.000	ml

Directions:

@ = Add after bringing up to volume

5 # = Add after sterilizing and cooling to temp.

Dissolve ingredients in polished D-I H<sub>2</sub>O in sequence

Adjust to pH 5.8

Bring up to volume with polished D-I H<sub>2</sub>O after adjusting pH

Sterilize and cool to 60°C.

10 #### = Dissolve 1.660 g of Calcium Chloride Dihydrate in 950.000 ml of polished D-I H<sub>2</sub>O. Then dissolve 4.629 of Ammonium Sulfate; 4.000 g of Potassium Phosphate Monobasic KH<sub>2</sub>PO<sub>4</sub>; 1.850 g of Magnesium Sulfate 7-H<sub>2</sub>O, MgSO<sub>4</sub>,

7H<sub>2</sub>O; and 28.300 g of Potassium Nitrate into sequence. Bring up to volume with polished D-I H<sub>2</sub>O.

### = Dissolve 3.000 g of Boric Acid; 10.000 g of Manganous Sulfate

Monohydrate; 0.250 g of Sodium Molybdate Dihydrate; and 0.750 g of Potassium

- 5 Iodide in polished D-I H<sub>2</sub>O in sequence. Bring up to volume with polished D-I H<sub>2</sub>O.

#### = Dissolve 3.700 g of Disodium EDTA Dihydrate and 2.790 g of Ferrous Sulfate 7-Hydrate into D-I H<sub>2</sub>O. Bring up to volume with D-I H<sub>2</sub>O.

Total Volume (L) = 1.00

## 605 J

Ingredient	Amount	Unit
D-I H <sub>2</sub> O	900.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	1.600	g
N6 Macronutrients 10X Stock ##	60.000	ml
Potassium Nitrate	1.680	g
B5H Minor Salts 1000X ###	0.600	ml
B5H Fe Na EDTA 100X ####	6.000	ml
Eriksson's Vitamin Mix (1000X SIGMA-1511)	0.400	ml
S & H Vitamin Mixture 100X Stock (S3766)	6.000	ml
Thiamine HCL 0.4mg/ml	0.500	ml
Sucrose	20.000	g
Glucose	0.600	g
2, 4-D 0.5mg/ml	1.600	ml
Gelrite @	2.000	g
Dicamba 1mg/ml #	1.200	ml
Silver Nitrate 2mg/ml #	0.425	ml
Bialaphos 1 mg/ml #	3.000	ml

Directions.

@ = Add after bringing up to volume

5 # = Add after sterilizing and cooling to temp.

Dissolve ingredients in polished D-I H<sub>2</sub>O in sequence

Adjust to pH 5.8

Bring up to volume with polished D-I H<sub>2</sub>O after adjusting pH

Sterilize and cool to 60°C.

10 ## = Dissolve 1.660 g of Calcium Chloride Dihydrate in 950.000 ml of polished D-I H<sub>2</sub>O. Then dissolve 4.629 of Ammonium Sulfate; 4.000 g of Potassium Phosphate Monobasic KH<sub>2</sub>PO<sub>4</sub>; 1.850 g of Magnesium Sulfate 7-H<sub>2</sub>O, MgSO<sub>4</sub>, 7H<sub>2</sub>O; and 28.300 g of Potassium Nitrate into sequence. Bring up to volume with polished D-I H<sub>2</sub>O.

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### = Dissolve 3.000 g of Boric Acid, 10.000 g of Manganous Sulfate Monohydrate, 0.250 g of Sodium Molybdate Dihydrate, and 0.750 g of Potassium Iodide in polished D-I H<sub>2</sub>O in sequence. Bring up to volume with polished D-I H<sub>2</sub>O.

- 5 #### = Dissolve 3.700 g of Disodium EDTA Dihydrate and 2.790 g of Ferrous Sulfate 7-Hydrate into D-I H<sub>2</sub>O. Bring up to volume with D-I H<sub>2</sub>O.

Total Volume (L) = 1.00

## 604 S

Ingredient	Amount	Unit
D-I H <sub>2</sub> O	800.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	1.600	g
N6 Macronutrients 10X Stock ##	60.000	ml
Potassium Nitrate	1.680	g
B5H Minor Salts 1000X ###	0.600	ml
B5H Fe Na EDTA 100X ####	6.000	ml
Eriksson's Vitamin Mix (1000X SIGMA-1511)	0.400	ml
S & H Vitamin Mixture 100X Stock (S3766)	6.000	ml
Thiamine.HCL 0.4mg/ml	0.500	ml
L-Proline	1.980	g
Casein Hydrolysate (acid)	0.300	g
Sucrose	120.000	g
Glucose	0.600	g
2, 4-D 0.5mg/ml	1.600	ml
Gelrite @	2.000	g
Dicamba 1mg/ml #	1.200	ml
Silver Nitrate 2mg/ml #	1.700	ml

Directions:

@ = Add after bringing up to volume

5 # = Add after sterilizing and cooling to temp.

Dissolve ingredients in polished D-I H<sub>2</sub>O in sequence

Adjust to pH 5.8

Bring up to volume with polished D-I H<sub>2</sub>O after adjusting pH

Sterilize and cool to 60°C.

10 #### = Dissolve 1.660 g of Calcium Chloride Dihydrate in 950.000 ml of polished D-I H<sub>2</sub>O. Then dissolve 4.629 of Ammonium Sulfate; 4.000 g of Potassium Phosphate Monobasic KH<sub>2</sub>PO<sub>4</sub>; 1.850 g of Magnesium Sulfate 7-H<sub>2</sub>O, MgSO<sub>4</sub>, 7H<sub>2</sub>O; and 28.300 g of Potassium Nitrate into sequence. Bring up to volume with polished D-I H<sub>2</sub>O.

## = Dissolve 3.000 g of Boric Acid; 10.000 g of Manganous Sulfate Monohydrate; 0.250 g of Sodium Molybdate Dihydrate; and 0.750 g of Potassium Iodide in 950.000 ml of polished D-I H<sub>2</sub>O in sequence. Bring up to volume with polished D-I H<sub>2</sub>O.

- 5 #### = Dissolve 3.700 g of Disodium EDTA Dihydrate and 2.790 g of Ferrous Sulfate 7-Hydrate into 950.000 ml of D-I H<sub>2</sub>O. Bring up to volume with D-I H<sub>2</sub>O.  
Total Volume (L) = 1.00

## 272 V

Ingredient	Amount	Unit
D-I H <sub>2</sub> O	950.000	ml
MS Salts (GIBCO 11117-074)	4.300	g
Myo-Inositol	0.100	g
MS Vitamins Stock Solution ##	5.000	ml
Sucrose	40.000	g
Bacto-Agar @	6.000	g

Directions:

@ = Add after bringing up to volume

- 5 Dissolve ingredients in polished D-I H<sub>2</sub>O in sequence

Adjust to pH 5.6

Bring up to volume with polished D-I H<sub>2</sub>O after adjusting pH

Sterilize and cool to 60°C.

## = Dissolve 0.100 g of Nicotinic Acid; 0.020 g of Thiamine.HCL; 0.100 g of

- 10 Pyridoxine.HCL; and 0.400 g of Glycine in 875.00 ml of polished D-I H<sub>2</sub>O in sequence. Bring up to volume with polished D-I H<sub>2</sub>O. Make in 400 ml portions. Thiamine.HCL & Pyridoxine.HCL are in Dark Desiccator. Store for one month, unless contamination or precipitation occur, then make fresh stock.

Total Volume (L) = 1.00

## 288 J

Ingredient	Amount	Unit
D-I H <sub>2</sub> O	950.000	ml
MS Salts	4.300	g
Myo-Inositol	0.100	g
MS Vitamins Stock Solution ##	5.000	ml
Zeatin .5mg/ml	1.000	ml
Sucrose	60.000	g
Gelrite @	3.000	g
Indole Acetic Acid 0.5 mg/ml #	2.000	ml
1mM Absissic Acid	1.000	ml
Bialaphos 1mg/ml #	3.000	ml

## Directions:

@ = Add after bringing up to volume

- 5 Dissolve ingredients in polished D-I H<sub>2</sub>O in sequence

Adjust to pH 5.6

Bring up to volume with polished D-I H<sub>2</sub>O after adjusting pH

Sterilize and cool to 60°C.

Add 3.5g/L of Gelrite for cell biology.

- 10 ## = Dissolve 0.100 g of Nicotinic Acid; 0.020 g of Thiamine.HCL; 0.100 g of Pyridoxine.HCL; and 0.400 g of Glycine in 875.00 ml of polished D-I H<sub>2</sub>O in sequence. Bring up to volume with polished D-I H<sub>2</sub>O. Make in 400 ml portions. Thiamine.HCL & Pyridoxine.HCL are in Dark Desiccator. Store for one month, unless contamination or precipitation occur, then make fresh stock.
- 15 Total Volume (L) = 1.00

## 560 L

Ingredient	Amount	Unit
D-I Water, Filtered	950.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	4.000	g
Eriksson's Vitamin Mix (1000X SIGMA-1511)	0.400	ml
Thiamine.HCL 0.4mg/ml	1.250	ml
Sucrose	20.000	g
2, 4-D 0.5mg/ml	2.000	ml
L-Proline	2.880	g
Gelrite @	2.000	g
Silver Nitrate 2mg/ml #	4.250	ml

Directions:

@ = Add after bringing up to volume

5 # = Add after sterilizing and cooling to temp.

Dissolve ingredients in D-I H<sub>2</sub>O in sequence

Adjust to pH 5.8 with KOH

Bring up to volume with D-I H<sub>2</sub>O

Sterilize and cool to room temp.

10 Total Volume (L) = 1.00

## 560 R

Ingredient	Amount	Unit
D-I Water, Filtered	950.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	4.000	g
Eriksson's Vitamin Mix (1000X SIGMA-1511)	1.000	ml
Thiamine.HCL 0.4mg/ml	1.250	ml
Sucrose	30.000	g
2, 4-D 0.5mg/ml	4.000	ml
Gelrite @	3.000	g
Silver Nitrate 2mg/ml #	0.425	ml
Bialaphos 1mg/ml #	3.000	ml

Directions:

- @ = Add after bringing up to volume
- 5 # = Add after sterilizing and cooling to temp.
- Dissolve ingredients in D-I H<sub>2</sub>O in sequence
- Adjust to pH 5.8 with KOH
- Bring up to volume with D-I H<sub>2</sub>O
- Sterilize and cool to room temp.
- 10 Total Volume (L) = 1.00

## 560 Y

Ingredient	Amount	Unit
D-I Water, Filtered	950.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	4.000	g
Eriksson's Vitamin Mix (1000X SIGMA-1511)	1.000	ml
Thiamine.HCL 0.4mg/ml	1.250	ml
Sucrose	120.000	g
2,4-D 0.5mg/ml	2.000	ml
L-Proline	2.880	g
Gelrite @	2.000	g
Silver Nitrate 2mg/ml #	4.250	ml

Directions:

@ = Add after bringing up to volume

5 # = Add after sterilizing and cooling to temp.

Dissolve ingredients in D-I H<sub>2</sub>O in sequence

Adjust to pH 5.8 with KOH

Bring up to volume with D-I H<sub>2</sub>O

Sterilize and cool to room temp.

10 \*\* Autoclave less time because of increased sucrose\*\*

Total Volume (L) = 1.00

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains.

All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically  
5 and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

## THAT WHICH IS CLAIMED:

1. A method for manipulating a metabolic pathway in a plant cell, said method comprising transforming a plant cell with at least one nucleotide sequence operably linked to a promoter that drives expression in a plant cell, wherein said nucleotide sequence is selected from the group consisting of:
- a) a nucleotide sequence encoding a limonene synthase protein,
  - b) a nucleotide sequence encoding a GPP synthase protein,
  - c) a nucleotide sequence encoding a carveol synthase protein,
  - 10 d) a nucleotide sequence encoding a S-linalool synthase protein,
  - e) a nucleotide sequence encoding a limonene synthase protein having the amino acid sequence set forth in SEQ ID NO:2,
  - f) a nucleotide sequence encoding a GPP synthase protein having the amino acid sequence set forth in SEQ ID NO:4,
  - 15 g) a nucleotide sequence encoding a carveol synthase protein having the amino acid sequence set forth in SEQ ID NO:6,
  - h) a nucleotide sequence encoding a S-linalool synthase protein having the amino acid sequence set forth in SEQ ID NO:8,
  - i) a nucleotide sequence encoding a limonene synthase protein as set forth in SEQ ID NO:1,
  - 20 j) a nucleotide sequence encoding a GPP synthase protein as set forth in SEQ ID NO:3,
  - k) a nucleotide sequence encoding a carveol synthase protein as set forth in SEQ ID NO:5,
  - 25 l) a nucleotide sequence encoding a S-linalool synthase protein as set forth in SEQ ID NO:7,
  - m) a nucleotide sequence corresponding to a portion of an antisense sequence for said nucleotide sequences of a, b, c, d, e, f, g, h, i, j, k or l.
- 30 2. The method of claim 1 wherein said plant cell is monocotyledonous.
3. The method of claim 2 wherein said monocot cell is a corn, wheat, rice, oat, rye or sorghum cell.

4. The method of claim 1 wherein said promoter is a constitutive promoter.

5. The method of claim 4 wherein said constitutive promoter is a ubiquitin promoter.

6. The method of claim 1 wherein said promoter is a tissue-specific promoter.

10

7. The method of claim 6 wherein said tissue-specific promoter is a root-specific promoter.

8. A method for manipulating a metabolic pathway in a plant cell, said method comprising transforming a plant cell with a first nucleotide sequence encoding a GPP synthase protein, and at least one nucleotide sequence which is selected from the group consisting of:

- a) a nucleotide sequence encoding a limonene synthase protein,
- b) a nucleotide sequence encoding a carveol synthase protein,
- 20 c) a nucleotide sequence encoding a S-linalool synthase protein,
- d) a nucleotide sequence encoding a limonene synthase protein having the amino acid sequence set forth in SEQ ID NO:2,
- e) a nucleotide sequence encoding a carveol synthase protein having the amino acid sequence set forth in SEQ ID NO:6,
- 25 f) a nucleotide sequence encoding a S-linalool synthase protein having the amino acid sequence set forth in SEQ ID NO:8,
- g) a nucleotide sequence encoding a limonene synthase protein as set forth in SEQ ID NO:1,
- h) A nucleotide sequence encoding a carveol synthase protein as forth in SEQ ID NO:5,
- 30 i) A nucleotide sequence encoding a S-linalool synthase protein as set forth in SEQ ID NO:7,

j) a nucleotide sequence corresponding to a portion of an antisense sequence for said nucleotide sequences of a, b, c, d, e, f, g, h or i; and wherein said first and said second nucleotide sequence are operably linked to a promoter that drives expression in a plant cell.

5

9. The method of claim 8, wherein said plant cell is co-transformed.

10. A method for creating or enhancing resistance to insects in a plant, said method comprising manipulating a metabolic pathway in a plant cell according to the method of claim 8, wherein said selected nucleotide sequence consists of a second nucleotide sequence encoding a limonene synthase protein, and further comprising regenerating stably transformed plants producing effective amounts of limonene.

15

11. A method for creating or enhancing resistance to insects in a plant, said method comprising manipulating a metabolic pathway in a plant cell according to the method of claim 8, wherein said selected nucleotide sequences consist of a second nucleotide sequence encoding a limonene synthase protein and a third nucleotide sequence encoding a carveol synthase protein; and further comprising regenerating stably transformed plants producing effective amounts of carveol.

25

12. A method for creating or enhancing resistance to insects in a plant, said method comprising manipulating a metabolic pathway in a plant cell according to the method of claim 8, wherein said selected nucleotide sequence consists of a second nucleotide sequence encoding a S-linalool synthase protein, and further comprising regenerating stably transformed plants producing effective amounts of S-linalool.

30

13. The method of any of the claims 10, 11, or 12 wherein said plant is monocotyledonous.

14. The method of claim 13 wherein said monocot plant is a corn plant  
5 (Z. mays L.).

15. The method of claim 14 wherein said insect is larvae of *Diabrotica* spp.

10 16. A method of producing limonene synthase and GPP synthase in a plant cell, said method comprising transforming a first plant cell with a nucleotide sequence encoding a limonene synthase protein operably linked to a promoter that drives expression in a plant cell,

transforming a second plant cell with a nucleotide sequence encoding a  
15 GPP synthase protein operably linked to a promoter that drives expression in a plant cell,

regenerating a first fertile transgenic plant from said first plant cell,  
regenerating a second fertile transgenic plant from said second plant cell,  
crossing said first and second fertile transgenic plants; and  
20 recovering progeny from said cross which express limonene synthase and GPP synthase.

17. A method of producing limonene synthase, GPP synthase, and carveol synthase in a plant cell, said method comprising  
25 transforming a first plant cell with a nucleotide sequence encoding a limonene synthase protein operably linked to a promoter that drives expression in a plant cell,

transforming a second plant cell with a nucleotide sequence encoding a  
GPP synthase protein operably linked to a promoter that drives expression in a  
30 plant cell,

transforming a third plant cell with a nucleotide sequence encoding a  
carveol synthase protein operably linked to a promoter that drives expression in a  
plant cell,

producing a first fertile transgenic plant from said first plant cell,  
producing a second fertile transgenic plant from said second plant cell,  
producing a third fertile transgenic plant from said third plant cell,  
performing a first cross between said first and second fertile transgenic  
5 plants,  
recovering progeny from said first cross,  
performing a second cross between said third fertile transgenic plant and  
said progeny of said first cross; and  
recovering progeny from said second cross which express limonene  
10 synthase, GPP synthase and carveol synthase.

18. The method of claim 17, wherein said second plant cell is  
transformed with a nucleotide sequence encoding a carveol synthase protein, and  
said third plant cell is transformed with a nucleotide sequence encoding GPP  
15 synthase.

19. A method of producing GPP synthase and S-linalool synthase in a  
plant cell, said method comprising  
transforming a first plant cell with a nucleotide sequence encoding a GPP  
20 synthase protein operably linked to a promoter that drives expression in a plant  
cell,  
transforming a second plant cell with a nucleotide sequence encoding a S-  
linalool synthase protein operably linked to a promoter that drives expression in a  
plant cell,  
25 producing a first fertile transgenic plant from said first plant cell,  
producing a second fertile transgenic plant from said second plant cell,  
crossing said first and second fertile transgenic plants; and  
recovering progeny from said cross which express GPP synthase and S-  
linalool synthase.

30 20. A transformed plant cell having a manipulated metabolic pathway  
by having stably incorporated into its genome at least one nucleotide sequence  
operably linked to a promoter that drives expression in a plant cell,

wherein said nucleotide sequence is selected from the group consisting of:

- a) a nucleotide sequence encoding a limonene synthase protein,
- b) a nucleotide sequence encoding a GPP synthase protein,
- c) a nucleotide sequence encoding a carveol synthase protein,
- 5 d) a nucleotide sequence encoding a S-linalool synthase protein,
- e) a nucleotide sequence encoding a limonene synthase protein having the amino acid sequence set forth in SEQ ID NO:2,
- f) a nucleotide sequence encoding a GPP synthase protein having the amino acid sequence set forth in SEQ ID NO:4,
- 10 g) a nucleotide sequence encoding a carveol synthase protein having the amino acid sequence set forth in SEQ ID NO:6,
- h) a nucleotide sequence encoding a S-linalool synthase protein having the amino acid sequence set forth in SEQ ID NO:8,
- i) a nucleotide sequence encoding a limonene synthase protein as set forth in SEQ ID NO:1,
- 15 j) a nucleotide sequence encoding a GPP synthase protein as set forth in SEQ ID NO:3,
- k) a nucleotide sequence encoding a carveol synthase protein as set forth in SEQ ID NO:5,
- 20 l) a nucleotide sequence encoding a S-linalool synthase protein as set forth in SEQ ID NO:7,
- m) a nucleotide sequence corresponding to a portion of an antisense sequence for said nucleotide sequences of a, b, c, d, e, f, g, h, i, k or l.

25 21. A transformed plant having a manipulated metabolic pathway by having stably incorporated into its genome at least one nucleotide sequence operably linked to a promoter that drives expression in a plant cell,

wherein said nucleotide sequence is selected from the group consisting of:

- a) a nucleotide sequence encoding a limonene synthase protein,
- 30 b) a nucleotide sequence encoding a GPP synthase protein,
- c) a nucleotide sequence encoding a carveol synthase protein,
- d) a nucleotide sequence encoding a S-linalool synthase protein,

- e) a nucleotide sequence encoding a limonene synthase protein having the amino acid sequence set forth in SEQ ID NO:2,
- f) a nucleotide sequence encoding a GPP synthase protein having the amino acid sequence set forth in SEQ ID NO:4,
- 5 g) a nucleotide sequence encoding a carveol synthase protein having the amino acid sequence set forth in SEQ ID NO:6,
- h) a nucleotide sequence encoding a S-linalool synthase protein having the amino acid sequence set forth in SEQ ID NO:8,
- i) a nucleotide sequence encoding a limonene synthase protein as set  
10 forth in SEQ ID NO:1,
- j) a nucleotide sequence encoding a GPP synthase protein as set forth in SEQ ID NO:3,
- k) a nucleotide sequence encoding a carveol synthase protein as set forth in SEQ ID NO:5,
- 15 l) a nucleotide sequence encoding a S-linalool synthase protein as set forth in SEQ ID NO:7,
- m) a nucleotide sequence corresponding to a portion of an antisense sequence for said nucleotide sequences of a, b, c, d, e, f, g, h, i, j, k or l.
- 20 22. The plant of claim 21 wherein said plant is monocotyledonous.
23. The plant of claim 22 wherein said monocot plant is a corn, wheat, rice, oat, rye or sorghum plant.
- 25 24. The plant of claim 21 wherein said promoter is a constitutive promoter.
25. The plant of claim 24 wherein said constitutive promoter is a ubiquitin promoter.
- 30 26. The plant of claim 21 wherein said promoter is a tissue-specific promoter.

27. The plant of claim 26 wherein said tissue-specific promoter is a root-specific promoter.

28. The plant of any of claims 24-27 wherein said plant is a dicot.

5

29. Seed of the plant of any of claims 21-27.

30. Seed of the plant of claim 28.

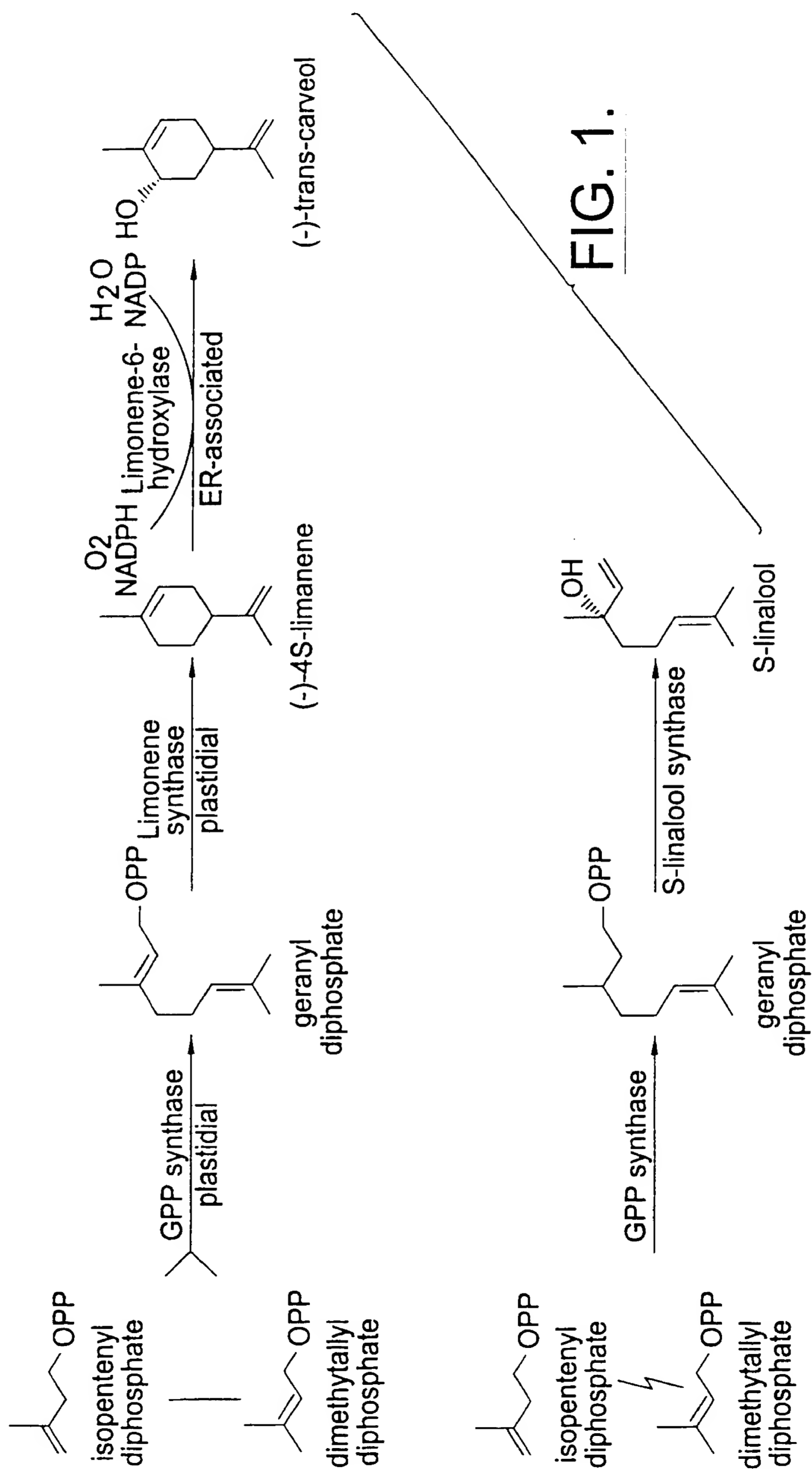


FIG. 1.

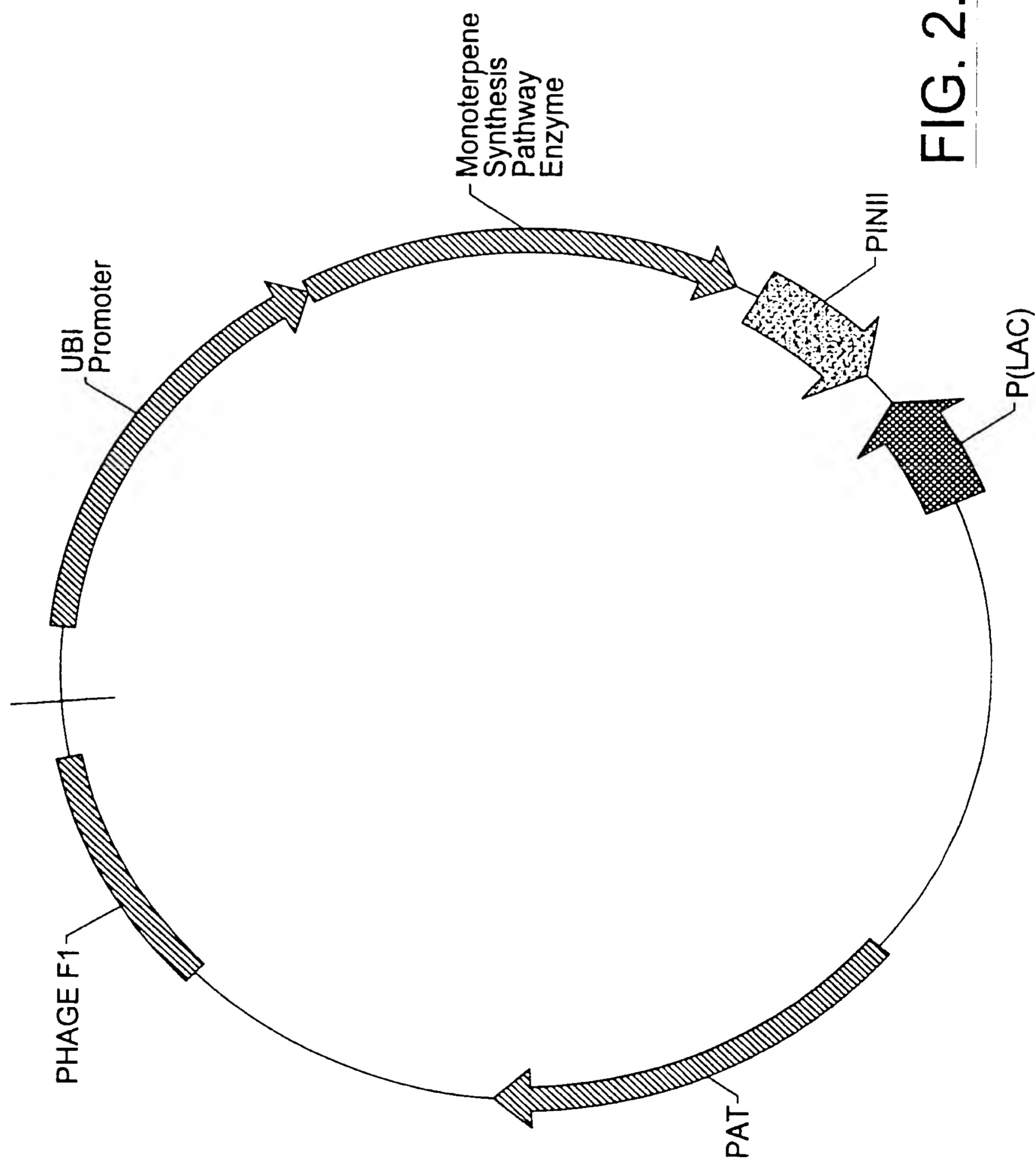


FIG. 2.

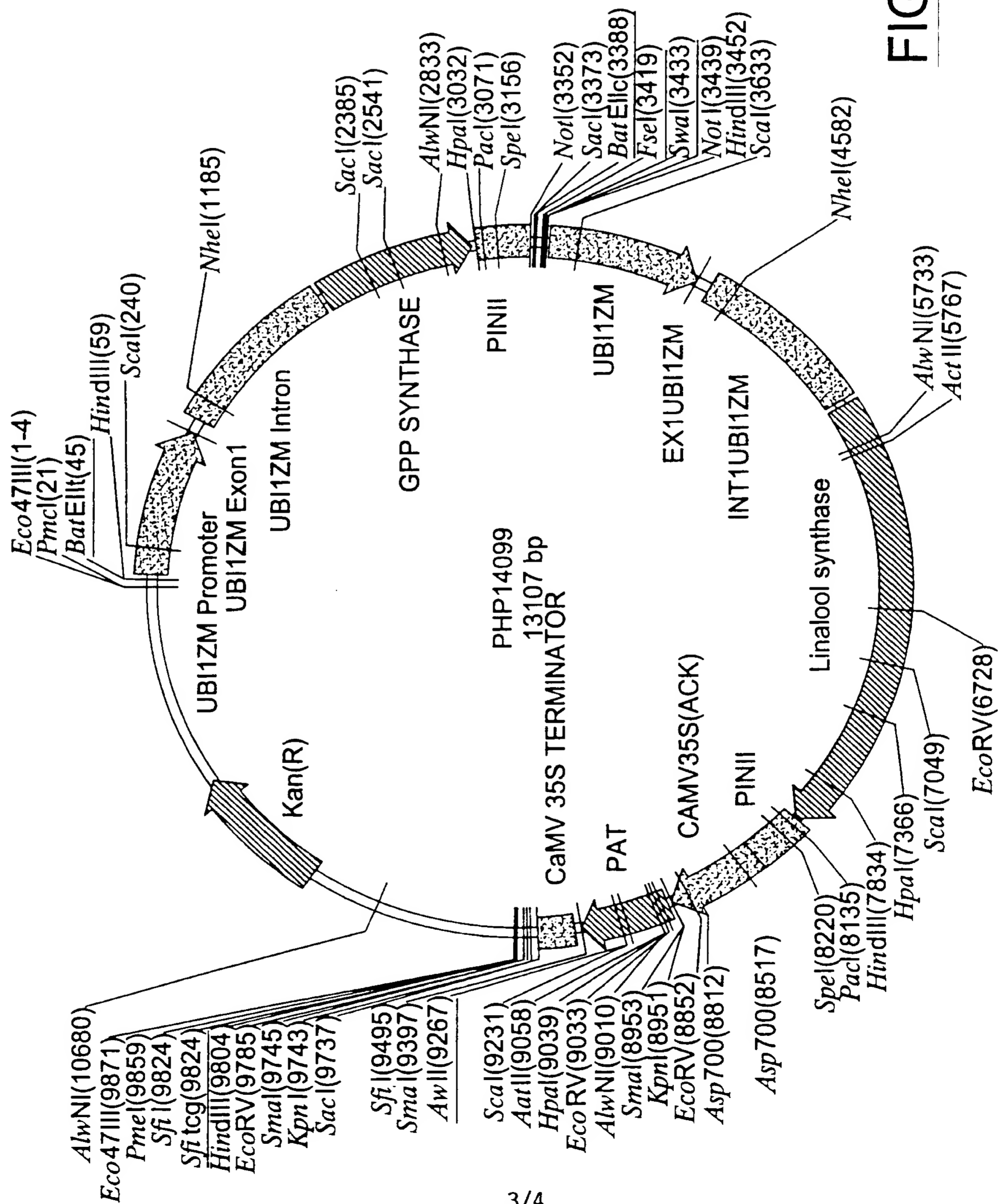


FIG. 3.

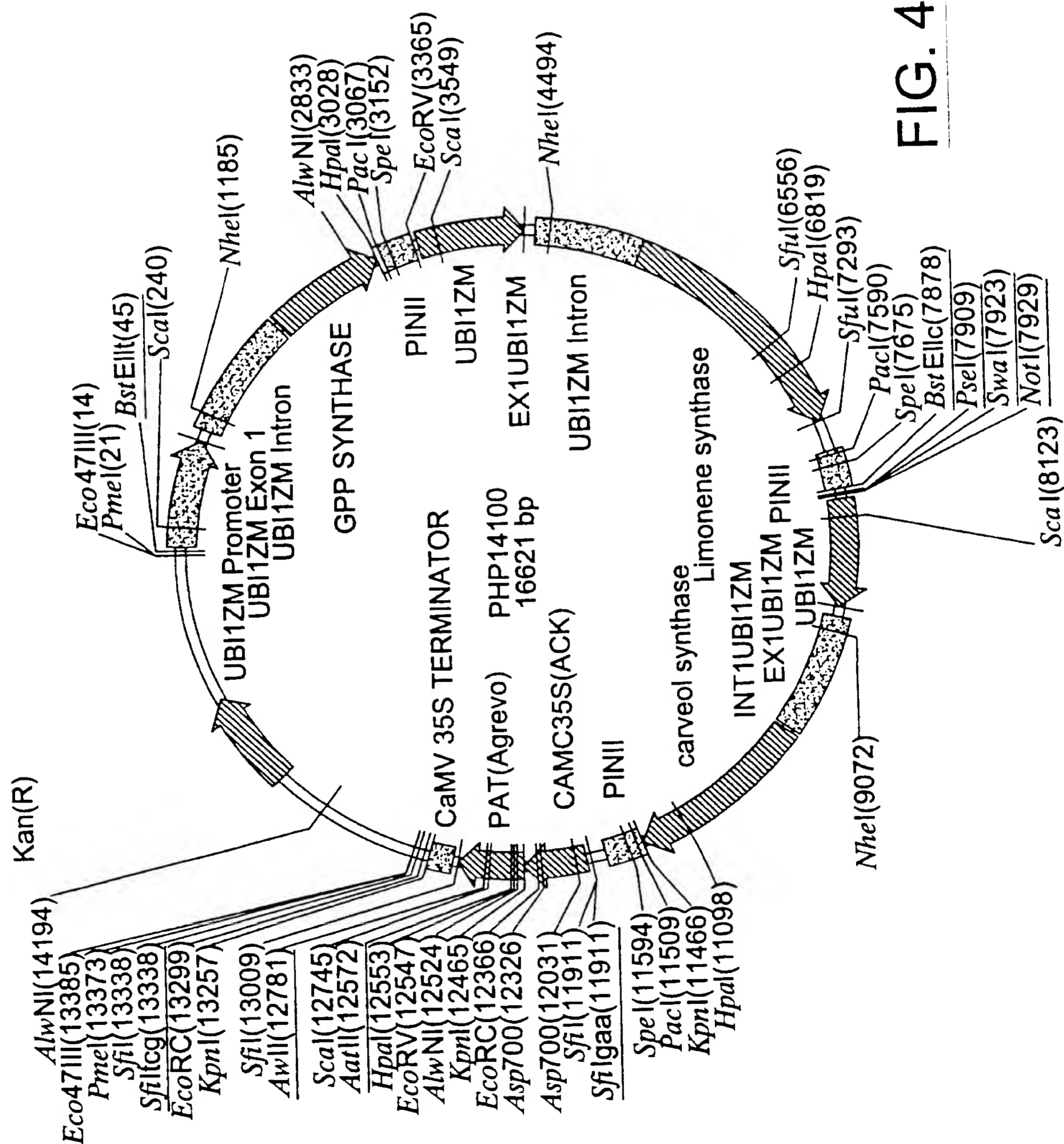


FIG. 4.

## SEQUENCE LISTING

<110> EuClaire Meyer, Terry  
Yalpani, Nasser

<120> Limonene and Other Downstream  
Metabolites of Geranyl Pyrophosphate for Insect Control in  
Plants

<130> 5718-65

<140> 09/172,339

<141> 1998-10-14

<150> 08/449,061

<151> 1995-05-24

<150> 08/153,544

<151> 1993-11-16

<150> 08/042,199

<151> 1993-04-02

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&lt;212&gt; FRT

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Asn Leu Thr Thr Cys Leu Gln Pro Ser His Phe Lys Ser Ser Pro Lys  
 20 25 30

Leu Leu Ser Ser Thr Asn Ser Ser Ser Arg Ser Arg Leu Arg Val Tyr  
 35 40 45

Cys Ser Ser Ser Gln Leu Thr Thr Glu Arg Arg Ser Gly Asn Tyr Asn  
 50 55 60

Pro Ser Arg Trp Asp Val Asn Phe Ile Gln Ser Leu Leu Ser Asp Tyr  
 65 70 75 80

Lys Glu Asp Lys His Val Ile Arg Ala Ser Glu Leu Val Thr Leu Val  
 85 90 95

Lys Met Glu Leu Glu Lys Glu Thr Asp Gln Ile Arg Gln Leu Glu Leu  
 100 105 110

Ile Asp Asp Leu Gln Arg Met Gly Leu Ser Asp His Phe Gln Asn Glu  
 115 120 125

Phe Lys Glu Ile Leu Ser Ser Ile Tyr Leu Asp His His Tyr Tyr Lys  
 130 135 140

Asn Pro Phe Pro Lys Glu Arg Asp Leu Tyr Ser Thr Ser Leu Ala  
 145 150 155 160

Phe Arg Leu Leu Arg Glu His Gly Phe Gln Val Ala Gln Glu Val Phe

$\langle 210 \rangle \quad 3$

<211> 1131  
 <212> DNA  
 <213> Mentha spicata

<220>  
 <221> misc\_feature  
 <222> (0)...(0)  
 <223> GPP Synthase

<221> CDS  
 <222> (6)...(944)

<400> 3

tcaaa atg gcc att aat ctc tcc cat atc aac tcc aaa aca tgt ttc cct	50
Met Ala Ile Asn Leu Ser His Ile Asn Ser Lys Thr Cys Phe Pro	
1 5 10 15	
ctc aaa aca aga tct gat ctc agc cgt tct tct tcc gcg cgt tgc atg	98
Leu Lys Thr Arg Ser Asp Leu Ser Arg Ser Ser Ser Ala Arg Cys Met	
20 25 30	
cca act gcc gcc gct gcc gcc ttc ccc act atc gcc acc gcc gcc caa	146
Pro Thr Ala Ala Ala Ala Phe Pro Thr Ile Ala Thr Ala Ala Gln	
35 40 45	
agt cag ccg tac tgg gcc gcc atc gag gcc gac ata gag aga tac ctg	194
Ser Gln Pro Tyr Trp Ala Ala Ile Glu Ala Asp Ile Glu Arg Tyr Leu	
50 55 60	
aag aaa tcc atc aca ata agg ccg ccg gag aca gtt ttc ggg ccc atg	242
Lys Lys Ser Ile Thr Ile Arg Pro Pro Glu Thr Val Phe Gly Pro Met	
65 70 75	
cac cac ctc acc ttc gcc gcc cca gcc acc gcc gcc tcc acc cta tgc	290
His His Leu Thr Phe Ala Ala Pro Ala Thr Ala Ala Ser Thr Leu Cys	
80 85 90 95	
ttg gcg gcg tgc gag ctc gtc ggc ggc gac cga agc caa gcc atg gca	338
Leu Ala Ala Cys Glu Leu Val Gly Gly Asp Arg Ser Gln Ala Met Ala	
100 105 110	
gcc gcg gcg gcg atc cat ctc gtg cac gcg gca gcc tac gtc cac gag	386
Ala Ala Ala Ala Ile His Leu Val His Ala Ala Ala Tyr Val His Glu	
115 120 125	
cac ctc cct cta acc gac ggg tcg agg ccc gta tcc aag ccc gca atc	434
His Leu Pro Leu Thr Asp Gly Ser Arg Pro Val Ser Lys Pro Ala Ile	
130 135 140	
cag cac aag tac ggc ccg aac gtc gag ctc ctc acc gga gac ggg att	482
Gln His Lys Tyr Gly Pro Asn Val Glu Leu Leu Thr Gly Asp Gly Ile	
145 150 155	
gtc ccg ttc ggg ttt gag ttg ctg gcc ggg tca gtg gac ccg gcc cga	530
Val Pro Phe Gly Phe Glu Leu Leu Ala Gly Ser Val Asp Pro Ala Arg	
160 165 170 175	

aca gac gac ccg gat agg att ctg aga gtt ata ata gag atc agt cgg 578  
 Thr Asp Asp Pro Asp Arg Ile Leu Arg Val Ile Ile Glu Ile Ser Arg  
 180 185 190  
 gcc ggc ggg ccg gag gga atg ata agc ggg ctg cat agg gaa gaa gaa 626  
 Ala Gly Gly Pro Glu Gly Met Ile Ser Gly Leu His Arg Glu Glu Glu  
 195 200 205  
 att gtt gat gga aat acg agt tta gac ttc att gaa tat gtg tgc aag 674  
 Ile Val Asp Gly Asn Thr Ser Leu Asp Phe Ile Glu Tyr Val Cys Lys  
 210 215 220  
 aaa aaa tac ggc gag atg cat gct tgc ggc gcg gct tgt gga gcc ata 722  
 Lys Lys Tyr Gly Glu Met His Ala Cys Gly Ala Ala Cys Gly Ala Ile  
 225 230 235  
 ttg ggc ggc gca gcc gag gag gag att cag aag ctg agg aat ttc ggg 770  
 Leu Gly Gly Ala Ala Glu Glu Glu Ile Gln Lys Leu Arg Asn Phe Gly  
 240 245 250 255  
 ctt tat cca gga act ctc aga gga atg atg gaa atg aaa aat tct cat 818  
 Leu Tyr Gln Gly Thr Leu Arg Gly Met Met Glu Met Lys Asn Ser His  
 260 265 270  
 caa tta att gat gag aat ata att gga aaa ttg aaa gaa ttg gct ctc 866  
 Gln Leu Ile Asp Glu Asn Ile Ile Gly Lys Leu Lys Glu Leu Ala Leu  
 275 280 285  
 gag gag ttg gga ggc ttc cac ggg aag aac gct gag ctg atg tcg agc 914  
 Glu Glu Leu Gly Gly Phe His Gly Lys Asn Ala Glu Leu Met Ser Ser  
 290 295 300  
 ctt gta gcc gag ccg agc ctt tac gcg gct tagagctatt cggatccttc 964  
 Leu Val Ala Glu Pro Ser Leu Tyr Ala Ala  
 305 310  
 attgcatttt catgcgacat cttcatatct atattgcata atattttttta agccagttat 1024  
 ttttttatta tgaatttttt taactgttat tgatttcgaa aatactgaca atcatctaaa 1084  
 ataaagtaaa tataagtaagg atgaaaaaaaa aaaaaaaaaa aaaaaaa 1131

&lt;210&gt; 4

&lt;211&gt; 313

&lt;212&gt; PRT

&lt;213&gt; Mentha spicata

&lt;400&gt; 4

Met Ala Ile Asn Leu Ser His Ile Asn Ser Lys Thr Cys Phe Pro Leu  
 1 5 10 15  
 Lys Thr Arg Ser Asp Leu Ser Arg Ser Ser Ser Ala Arg Cys Met Pro  
 20 25 30  
 Thr Ala Ala Ala Ala Phe Pro Thr Ile Ala Thr Ala Ala Gln Ser  
 35 40 45  
 Gln Pro Tyr Trp Ala Ala Ile Glu Ala Asp Ile Glu Arg Tyr Leu Lys  
 50 55 60  
 Lys Ser Ile Thr Ile Arg Pro Pro Glu Thr Val Phe Gly Pro Met His  
 65 70 75 80  
 His Leu Thr Phe Ala Ala Pro Ala Thr Ala Ala Ser Thr Leu Cys Leu

WO 00/22150

65 90 95  
 Ala Ala Cys Glu Leu Val Gly Gly Asp Arg Ser Gln Ala Met Ala Ala  
 100 105 110  
 Ala Ala Ala Ile His Leu Val His Ala Ala Ala Tyr Val His Glu His  
 115 120 125  
 Leu Pro Leu Thr Asp Gly Ser Arg Pro Val Ser Lys Pro Ala Ile Gln  
 130 135 140  
 His Lys Tyr Gly Pro Asn Val Glu Leu Leu Thr Gly Asp Gly Ile Val  
 145 150 155 160  
 Pro Phe Gly Phe Glu Leu Leu Ala Gly Ser Val Asp Pro Ala Arg Thr  
 165 170 175  
 Asp Asp Pro Asp Arg Ile Leu Arg Val Ile Ile Glu Ile Ser Arg Ala  
 180 185 190  
 Gly Gly Pro Glu Gly Met Ile Ser Gly Leu His Arg Glu Glu Glu Ile  
 195 200 205  
 Val Asp Gly Asn Thr Ser Leu Asp Phe Ile Glu Tyr Val Cys Lys Lys  
 210 215 220  
 Lys Tyr Gly Glu Met His Ala Cys Gly Ala Ala Cys Gly Ala Ile Leu  
 225 230 235 240  
 Gly Gly Ala Ala Glu Glu Glu Ile Gln Lys Leu Arg Asn Phe Gly Leu  
 245 250 255  
 Tyr Gln Gly Thr Leu Arg Gly Met Met Glu Met Lys Asn Ser His Gln  
 260 265 270  
 Leu Ile Asp Glu Asn Ile Ile Gly Lys Leu Lys Glu Leu Ala Leu Glu  
 275 280 285  
 Glu Leu Gly Gly Phe His Gly Lys Asn Ala Glu Leu Met Ser Ser Leu  
 290 295 300  
 Val Ala Glu Pro Ser Leu Tyr Ala Ala  
 305 310

<210> 5  
 <211> 1762  
 <212> DNA  
 <213> *Mentha spicata*

<220>  
 <221> misc\_feature  
 <222> (0)...(0)  
 <223> Carveol Synthase

<221> CDS  
 <222> (20)...(1507)

<400> 5  
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 Met Glu Leu Asp Leu Leu Ser Ala Ile Ile Ile 10  
 1 5  
 ctt gtg gca acc tac atc gta tcc ctc cta atc aac caa tgg cga aaa 100  
 Leu Val Ala Thr Tyr Ile Val Ser Leu Leu Ile Asn Gln Trp Arg Lys 25  
 15 20  
 tcg aaa tcc caa caa aac cta cct ccg agc cct ccg aag ctg ccg gtg 148  
 Ser Lys Ser Gln Gln Asn Leu Pro Pro Ser Pro Pro Lys Leu Pro Val 40  
 30 35  
 atc ggc cac ctc cac ttc ctg tgg gga ggg ctt ccc cag cac gtg ttt 196

Ile	Gly	His	Leu	His	Phe	Leu	Trp	Gly	Gly	Leu	Pro	Gln	His	Val	Phe		
	45					50					55						
agg	agc	ata	gcc	cag	aag	tac	ggg	ccg	gtg	gcg	cac	gtg	cag	ctg	gga	244	
Arg	Ser	Ile	Ala	Gln	Lys	Tyr	Gly	Pro	Val	Ala	His	Val	Gln	Leu	Gly		
	60				65				70						75		
gaa	gtg	tac	tgc	gtg	gtg	ctg	tgc	tgc	gcg	gag	gca	gcg	ccg	cag	gcg	292	
Glu	Val	Tyr	Ser	Val	Val	Leu	Ser	Ser	Ala	Glu	Ala	Ala	Pro	Gln	Ala		
				80					85					90			
atg	aag	gtg	ctg	gac	ccg	aac	ttc	gcc	gac	cgg	ttc	gac	ggc	atc	ggg	340	
Met	Lys	Val	Leu	Asp	Pro	Asn	Phe	Ala	Asp	Arg	Phe	Asp	Gly	Ile	Gly		
			95					100					105				
tcc	agg	acc	atg	tgg	tac	gac	aaa	gat	gac	atc	atc	ttc	agc	cct	tac	388	
Ser	Arg	Thr	Met	Trp	Tyr	Asp	Lys	Asp	Asp	Ile	Ile	Phe	Ser	Pro	Tyr		
		110					115					120					
aac	gat	cac	tgg	cgc	cag	atg	cgg	agg	atc	tgc	gtg	aca	gag	ctg	ctg	436	
Asn	Asp	His	Trp	Arg	Gln	Met	Arg	Arg	Ile	Cys	Val	Thr	Glu	Leu	Leu		
	125					130					135						
agc	ccg	aag	aac	gtc	agg	tcc	ttc	ggg	tac	ata	agg	cag	gag	gag	atc	484	
Ser	Pro	Lys	Asn	Val	Arg	Ser	Phe	Gly	Tyr	Ile	Arg	Gln	Glu	Glu	Ile		
	140				145					150					155		
gag	cgc	ctc	atc	cgg	ctg	ctc	ggg	tgc	tgc	ggc	gga	gcg	ccg	gtc	gac	532	
Glu	Arg	Leu	Ile	Arg	Leu	Leu	Gly	Ser	Ser	Gly	Gly	Ala	Pro	Val	Asp		
				160					165					170			
gtg	acg	gag	gag	gtg	tgc	aag	atg	tgc	tgt	gtc	gtc	gtg	tgc	agg	gcg	580	
Val	Thr		Glu	Glu	Val	Ser	Lys	Met	Ser	Cys	Val	Val	Val	Cys	Arg	Ala	
				175					180					185			
gcg	ttc	ggg	agt	gtg	ctc	aag	gac	cag	ggt	tgc	ttg	gcg	gag	ttg	gtg	628	
Ala	Phe	Gly	Ser	Val	Leu	Lys	Asp	Gln	Gly	Ser	Leu	Ala	Glu	Leu	Val		
		190					195					200					
aag	gag	tgc	ctg	gca	ttg	gcg	tcc	ggg	ttt	gag	ctg	gcg	gat	ctc	tac	676	
Lys	Glu	Ser	Leu	Ala	Leu	Ala	Ser	Gly	Phe	Glu	Leu	Ala	Asp	Leu	Tyr		
	205					210					215						
cct	tcc	tca	tgg	ctc	ctc	aac	ctg	ctt	agc	ttg	aac	aag	tac	agg	ttg	724	
Pro	Ser	Ser	Trp	Leu	Leu	Asn	Leu	Leu	Ser	Leu	Asn	Lys	Tyr	Arg	Leu		
					225					230					235		
cag	agg	atg	cgc	cgc	cgc	ctc	gat	cac	atc	ctt	gat	ggg	ttc	ctg	gag	772	
Gln	Arg	Met	Arg	Arg	Arg	Leu	Asp	His	Ile	Leu	Asp	Gly	Phe	Leu	Glu		
				240					245					250			
gag	cat	agg	gag	aag	aag	agc	ggc	gac	ttt	gga	ggc	gag	gac	atc	gtc	820	
Glu	His	Arg	Glu	Lys	Lys	Ser	Gly	Asp	Phe	Gly	Gly	Glu	Asp	Ile	Val		
			255					260					265				
gac	gtt	ctt	ttc	agg	atg	cag	ccg	ggc	agc	gac	agc	aaa	att	ccc	att	868	
Asp	Val	Leu	Phe	Arg	Met	Gln	Pro	Gly	Ser	Asp	Ser	Lys	Ile	Pro	Ile		

270	275	280	
act tcc aat tgc atc aag ggt ttc att ttc gac acc ttc tcc gcg gga Thr Ser Asn Cys Ile Lys Gly Phe Ile Phe Asp Thr Phe Ser Ala Gly 285 290 295			916
gct gaa acg tct tgc acg acc atc tca tgg gcg ttg tgc gaa ctg atg Ala Glu Thr Ser Ser Thr Thr Ile Ser Trp Ala Leu Ser Glu Leu Met 300 305 310 315			964
agg aat ccg gcg aag atg gcc aag gtg cag gcg gag gta aga gag gcg Arg Asn Pro Ala Lys Met Ala Lys Val Gln Ala Glu Val Arg Glu Ala 320 325 330			1012
ctc aag gga aag aca gtc gtg gat ttg agc gag gtg caa gag cta aaa Leu Lys Gly Lys Thr Val Val Asp Leu Ser Glu Val Gln Glu Leu Lys 335 340 345			1060
tac ctg aga tgc gtg tta aag gag act ctg agg ctg cac cct ccc ttt Tyr Leu Arg Ser Val Leu Lys Glu Thr Leu Arg Leu His Pro Pro Phe 350 355 360			1108
cca tta atc cca aga caa tcc agg gaa gaa tgc gag gtt aac ggg tac Pro Leu Ile Pro Arg Gln Ser Arg Glu Glu Cys Glu Val Asn Gly Tyr 365 370 375			1156
acg att ccg gcc aaa act aga atc ttc atc aac gtc tgg gct atc gga Thr Ile Pro Ala Lys Thr Arg Ile Phe Ile Asn Val Trp Ala Ile Gly 380 385 390 395			1204
agg gat ccc caa tac tgg gaa gat ccc gac acc ttc cgc cct gag aga Arg Asp Pro Gln Tyr Trp Glu Asp Pro Asp Thr Phe Arg Pro Glu Arg 400 405 410			1252
ttc gat gag gtt tcc agg gat ttc atg gga aac gat ttc gag ttc atc Phe Asp Glu Val Ser Arg Asp Phe Met Gly Asn Asp Phe Glu Phe Ile 415 420 425			1300
cca ttc ggg gcg ggt cga aga atc tgc ccc ggt tta cat ttc ggg ctg Pro Phe Gly Ala Gly Arg Arg Ile Cys Pro Gly Leu His Phe Gly Leu 430 435 440			1348
gca aat gtt gag atc cca ttg gcg caa ctg ctc tac cac ttc gac tgg Ala Asn Val Glu Ile Pro Leu Ala Gln Leu Leu Tyr His Phe Asp Trp 445 450 455			1396
aaa ttg cca caa gga atg act gat gcc gac ttg gca ctg acg gag acc Lys Leu Pro Gln Gly Met Thr Asp Ala Asp Leu Ala Leu Thr Glu Thr 460 465 470 475			1444
cca ggt ctt tct ggg cca aaa aag aaa aat gtt tgc ttg gtt ccc aca Pro Gly Leu Ser Gly Pro Lys Lys Lys Asn Val Cys Leu Val Pro Thr 480 485 490			1492
ctc tat aaa agt cct taaccactaa gaagtttagca taataagaca tctaaaattg Leu Tyr Lys Ser Pro 495			1547

tcataatcat ctaattattg ttacacttct tctatcatgt cattttgaga agtgtcttat 1607  
 agaggtggcc acggttccgg ttccagttcg gaagcggaac cgaaccatca gttacgggttc 1667  
 tcagcaagaa gcgaaccgtc ccgccccccc tactgtgttt gagatataaa acacataaaa 1727  
 taaaataaaa aaaacgctat ttttttttaa aaaaa 1762

&lt;210&gt; 6

&lt;211&gt; 496

&lt;212&gt; PRT

<213> *Mentha spicata*

&lt;400&gt; 6

Met	Glu	Leu	Asp	Leu	Leu	Ser	Ala	Ile	Ile	Ile	Leu	Val	Ala	Thr	Tyr
1				5					10					15	
Ile	Val	Ser	Leu	Leu	Ile	Asn	Gln	Trp	Arg	Lys	Ser	Lys	Ser	Gln	Gln
			20					25					30		
Asn	Leu	Pro	Pro	Ser	Pro	Pro	Lys	Leu	Pro	Val	Ile	Gly	His	Leu	His
		35					40					45			
Phe	Leu	Trp	Gly	Gly	Leu	Pro	Gln	His	Val	Phe	Arg	Ser	Ile	Ala	Gln
	50					55					60				
Lys	Tyr	Gly	Pro	Val	Ala	His	Val	Gln	Leu	Gly	Glu	Val	Tyr	Ser	Val
65					70					75					80
Val	Leu	Ser	Ser	Ala	Glu	Ala	Ala	Pro	Gln	Ala	Met	Lys	Val	Leu	Asp
			85						90					95	
Pro	Asn	Phe	Ala	Asp	Arg	Phe	Asp	Gly	Ile	Gly	Ser	Arg	Thr	Met	Trp
			100					105						110	
Tyr	Asp	Lys	Asp	Asp	Ile	Ile	Phe	Ser	Pro	Tyr	Asn	Asp	His	Trp	Arg
	115						120					125			
Gln	Met	Arg	Arg	Ile	Cys	Val	Thr	Glu	Leu	Leu	Ser	Pro	Lys	Asn	Val
	130					135						140			
Arg	Ser	Phe	Gly	Tyr	Ile	Arg	Gln	Glu	Glu	Ile	Glu	Arg	Leu	Ile	Arg
145					150					155					160
Leu	Leu	Gly	Ser	Ser	Gly	Gly	Ala	Pro	Val	Asp	Val	Thr	Glu	Glu	Val
			165						170					175	
Ser	Lys	Met	Ser	Cys	Val	Val	Val	Cys	Arg	Ala	Ala	Phe	Gly	Ser	Val
		180						185					190		
Leu	Lys	Asp	Gln	Gly	Ser	Leu	Ala	Glu	Leu	Val	Lys	Glu	Ser	Leu	Ala
	195						200					205			
Leu	Ala	Ser	Gly	Phe	Glu	Leu	Ala	Asp	Leu	Tyr	Pro	Ser	Ser	Trp	Leu
	210					215					220				
Leu	Asn	Leu	Leu	Ser	Leu	Asn	Lys	Tyr	Arg	Leu	Gln	Arg	Met	Arg	Arg
225					230					235					240
Arg	Leu	Asp	His	Ile	Leu	Asp	Gly	Phe	Leu	Glu	Glu	His	Arg	Glu	Lys
			245						250					255	
Lys	Ser	Gly	Asp	Phe	Gly	Gly	Glu	Asp	Ile	Val	Asp	Val	Leu	Phe	Arg
		260						265					270		
Met	Gln	Pro	Gly	Ser	Asp	Ser	Lys	Ile	Pro	Ile	Thr	Ser	Asn	Cys	Ile
	275						280					285			
Lys	Gly	Phe	Ile	Phe	Asp	Thr	Phe	Ser	Ala	Gly	Ala	Glu	Thr	Ser	Ser
	290					295					300				
Thr	Thr	Ile	Ser	Trp	Ala	Leu	Ser	Glu	Leu	Met	Arg	Asn	Pro	Ala	Lys
305					310					315					320
Met	Ala	Lys	Val	Gln	Ala	Glu	Val	Arg	Glu	Ala	Leu	Lys	Gly	Lys	Thr
			325						330					335	
Val	Val	Asp	Leu	Ser	Glu	Val	Gln	Glu	Leu	Lys	Tyr	Leu	Arg	Ser	Val
		340						345					350		
Leu	Lys	Glu	Thr	Leu	Arg	Leu	His	Pro	Pro	Phe	Pro	Leu	Ile	Pro	Arg

	355		360		365										
Gln	Ser	Arg	Glu	Glu	Cys	Glu	Val	Asn	Gly	Tyr	Thr	Ile	Pro	Ala	Lys
	370					375					380				
Thr	Arg	Ile	Phe	Ile	Asn	Val	Trp	Ala	Ile	Gly	Arg	Asp	Pro	Gln	Tyr
385					390					395					400
Trp	Glu	Asp	Pro	Asp	Thr	Phe	Arg	Pro	Glu	Arg	Phe	Asp	Glu	Val	Ser
				405					410					415	
Arg	Asp	Phe	Met	Gly	Asn	Asp	Phe	Glu	Phe	Ile	Pro	Phe	Gly	Ala	Gly
			420					425					430		
Arg	Arg	Ile	Cys	Pro	Gly	Leu	His	Phe	Gly	Leu	Ala	Asn	Val	Glu	Ile
		435					440						445		
Pro	Leu	Ala	Gln	Leu	Leu	Tyr	His	Phe	Asp	Trp	Lys	Leu	Pro	Gln	Gly
	450					455					460				
Met	Thr	Asp	Ala	Asp	Leu	Ala	Leu	Thr	Glu	Thr	Pro	Gly	Leu	Ser	Gly
465					470					475					480
Pro	Lys	Lys	Lys	Asn	Val	Cys	Leu	Val	Pro	Thr	Leu	Tyr	Lys	Ser	Pro
				485					490					495	

&lt;210&gt; 7

&lt;211&gt; 2681

&lt;212&gt; DNA

&lt;213&gt; Clarkia breweri

&lt;220&gt;

&lt;221&gt; misc\_feature

&lt;222&gt; (0)...(0)

&lt;223&gt; S-Linalool Synthase

&lt;221&gt; CDS

&lt;222&gt; (28)...(2637)

&lt;400&gt; 7

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Met Gln Leu Ile Thr Asn Phe Ser Ser	
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tca tca tca gaa ttg cag ttt ctt gtg gat aag gtt aag aga gaa tca	102
Ser Ser Ser Glu Leu Gln Phe Leu Val Asp Lys Val Lys Arg Glu Ser	
10 15 20 25	
ttg tct tct tca tca tct aat act cag aat ttg ttt ctc tca act tca	150
Leu Ser Ser Ser Ser Ser Asn Thr Gln Asn Leu Phe Leu Ser Thr Ser	
30 35 40	
cct tat gac act gct tgg ctc gcc ctt atc cct cat cct cat cat cac	198
Pro Tyr Asp Thr Ala Trp Leu Ala Leu Ile Pro His Pro His His His	
45 50 55	
cat cac cat ggc cga ccc atg ttt gaa aaa tgt ctg caa tgg att ctc	246
His His His Gly Arg Pro Met Phe Glu Lys Cys Leu Gln Trp Ile Leu	
60 65 70	
cat aac cag aca cca caa ggt ttc tgg gca gca gct ggt gac aat att	294
His Asn Gln Thr Pro Gln Gly Phe Trp Ala Ala Ala Gly Asp Asn Ile	
75 80 85	
tcc gac acc gac gat gac gtc acc ctg gat tgt ctt cta tca acc ttg	342

Ser 90	Asp	Thr	Asp	Asp	Asp 95	Val	Thr	Leu	Asp	Cys 100	Leu	Leu	Ser	Thr	Leu 105	
gct	tgc	tta	gtt	gca	ctc	aaa	agg	tgg	cag	ctt	gct	ccc	gac	atg	att	390
Ala	Cys	Leu	Val	Ala	Leu	Lys	Arg	Trp	Gln	Leu	Ala	Pro	Asp	Met	Ile	
				110					115					120		
cat	aaa	gga	ttg	gaa	ttt	gta	aat	aga	aac	aca	gag	aga	ctt	gta	atg	438
His	Lys	Gly	Leu	Glu	Phe	Val	Asn	Arg	Asn	Thr	Glu	Arg	Leu	Val	Met	
			125					130					135			
aag	cag	aag	ccg	agc	gac	gtt	cct	cgt	tgg	ttc	acc	atc	atg	ttc	ccg	486
Lys	Gln	Lys	Pro	Ser	Asp	Val	Pro	Arg	Trp	Phe	Thr	Ile	Met	Phe	Pro	
			140					145					150			
gcg	atg	ctc	gag	ctt	ccc	gga	gct	tcc	agt	ctc	cga	gtc	gat	ttc	agc	534
Ala	Met	Leu	Glu	Leu	Ala	Gly	Ala	Ser	Ser	Leu	Arg	Val	Asp	Phe	Ser	
	155					160					165					
gag	aat	ctt	aac	aga	atc	ttg	gtg	gaa	cta	tct	caa	aat	agg	gat	gat	582
Glu	Asn	Leu	Asn	Arg	Ile	Leu	Val	Glu	Leu	Ser	Gln	Asn	Arg	Asp	Asp	
170					175					180					185	
att	ctc	aca	agg	gag	gaa	gtt	gat	gag	aag	aag	caa	tac	tca	cca	ttg	630
Ile	Leu	Thr	Arg	Glu	Glu	Val	Asp	Glu	Lys	Lys	Gln	Tyr	Ser	Pro	Leu	
				190					195					200		
cta	cta	ttt	cta	gaa	gca	ttg	cct	gca	caa	tcc	tat	gac	aat	gat	gtt	678
Leu	Leu	Phe	Leu	Glu	Ala	Leu	Pro	Ala	Gln	Ser	Tyr	Asp	Asn	Asp	Val	
			205					210					215			
cta	aag	caa	att	ata	gac	aag	aac	ttg	agc	aat	gat	ggt	tct	tta	ttg	726
Leu	Lys	Gln	Ile	Ile	Asp	Lys	Asn	Leu	Ser	Asn	Asp	Gly	Ser	Leu	Leu	
		220					225					230				
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 Thr Gln Asn Leu Phe Leu Ser Thr Ser Pro Tyr Asp Thr Ala Trp Leu  
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 Phe Glu Lys Cys Leu Gln Trp Ile Leu His Asn Gln Thr Pro Gln Gly  
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Ser	Ser	Asn	Ile	Phe	Asp	Thr	Asp	Thr	Asp	Leu	Leu	Leu	Asp	Ile	His		
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Ile	Lys	Leu	Cys	Phe	Ser												
865					870												

## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/Us 99/23180

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/82 C12N9/88 C12N9/10 C12N9/00 C12N15/11  
A01H5/00

According to international Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 37102 A (PIONEER HI BRED INT) 28 November 1996 (1996-11-28)  the whole document ---	1-11, 13-16, 20-30
X	WO 94 22304 A (PIONEER HI BRED INT) 13 October 1994 (1994-10-13) the whole document ---	1-7, 20-30
X	WO 95 11913 A (UNIV WASHINGTON) 4 May 1995 (1995-05-04) the whole document ---	1-7, 20-30
	--- -/-	



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents:

"A" document defining the general state of the art which is not  
- considered to be of particular relevance

"E" earlier document but published on or after the international  
filing date

"L" document which may throw doubts on priority claim(s) or  
- which is cited to establish the publication date of another  
claim(s) or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or  
- other means

"P" document published prior to the international filing date but  
- later than the priority date claimed

"T" later document published after the international filing date  
or priority date and not in conflict with the application but  
cited to understand the principle or theory underlying the  
invention

"X" document of particular relevance: the claimed invention  
cannot be considered novel or cannot be considered to  
involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention  
cannot be considered to involve an inventive step when the  
document is combined with one or more other such docu-  
ments, such combination being obvious to a person skilled  
in the art

"Z" document member of the same patent family

Date of the actual completion of the international search

5 April 2000

Date of making of the international search report

25. 07. 00

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentkanal 2  
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Fax: (+31-70) 340-3016

Authorized officer

Oderwald, H

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 99/23180

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		Relevant to claim No.
Category *	Citation of document, with indication, where appropriate, of the relevant passages	
A	<p>COLBY S M ET AL: "4S-LIMONENE SYNTHASE FROM THE OIL GLANDS OF SPEARMINT (MENTHA SPICATA)" JOURNAL OF BIOLOGICAL CHEMISTRY, US, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 268, no. 31, 5 November 1993 (1993-11-05), pages 23016-23024, XP002914702 ISSN: 0021-9258 the whole document -----</p>	

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 99/23180

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

SEE ADDITIONAL SHEET

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
  
1-11, 13-18, 20-30 - partially

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: {1-11, 13-18, 20-30 partially}

A method for manipulating a metabolic pathway in a plant cell comprising transforming a plant cell with a limonene synthase-encoding nucleotide sequence set forth in SEQ ID NO: 1 and coding for a protein set forth in SEQ ID NO: 2. A method for creating or enhancing resistance to insects in a plant, a method for producing limonene synthase in a plant cell, a transformed plant cell and plant having a manipulated metabolic pathway comprising said limonene synthase.

2. Claims: {1-30 partially}

same as invention 1 but comprising a GPP synthase as set forth in SEQ ID NO: 3 and 4.

3. Claims: {1-9, 11, 13-15, 17, 18, 20-30 partially}

same as in invention but comprising a carveol synthase as set forth in SEQ ID NO: 5 and 6.

4. Claims: {1-9, 12-15, 19-30 partially}

same as invention 1 but comprising an S-linalool synthase as set forth in SEQ ID NO: 7 and 8.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/23180

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		AU 5879496 A	11-12-1996
		BR 9609204 A	11-05-1999
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		CA 2159735 A	13-10-1994
		DE 69420957 D	04-11-1999
		DE 69420957 T	13-01-2000
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		EP 0730603 A	11-09-1996
		US 5871988 A	16-02-1999
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